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THE BOTANICAL REVIEW

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PHOTOSYNTHESIS [1]

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INTRODUCTION

As our knowledge of photosynthesis enlarges, it becomes increasingly apparent, despite the multiplication of complexities, that the field may be divided without undue difficulties into two areas of research: photochemical and biosynthetic—in accord with the original nomenclature. One may speak with some definity in each of these areas, but mechanisms within their region of contact are still largely hypothetical.

It is customary to utilize photosynthesis as an example of the comparative biochemical aproach, i.e., to show the similarity of the process in the different photosynthetic groups of plants—*a*) the autotrophic chlorophyll-*a* plants which evolve O_2 during photosynthesis; *b*) the non-O_2-evolving autotrophic, but sulfide-requiring chlorophyll-*c*-containing, bacteria (the "green sulfur" bacteria); *c*) the semi-autotrophic purple bacteriochlorophyll microorganisms (Thiorhodaceae) which, like the *c* bacteria, do not evolve O_2 during photosynthesis, but which may utilize some organic compounds; and *d*) the purple, non-sulfur-utilizing bacteriochlorophyll-containing microorganisms (Athiorhodaceae) which generally require both organic donor and vitamins, utilizing light essentially as a catalyst, or, at best only as a partial source of energy.

The chemistry of CO_2 fixation has, in fact, shown these different classes to have essentially the same pattern of carbon assimilation. Then where does the difference in their requirements arise?

[1] Contribution No. 487 from the Department of Botany and Plant Pathology and the Institute for Atomic Research, Iowa State College, Ames, Iowa. This work has been supported in part by the National Science Foundation.

Naively, one may state that it is in either the pathway of the "A" or the "H", in the generalized equation

$$CO_2 + 2\,AH_2 \rightarrow (CH_2O) + H_2O + 2\,A,$$

where the "A" may be S or O (assuming the photochemical processes to be equivalent). One may question the actual generality of the equation as written, since certainly the general equation for chlorophyll-*a*-containing plants requires the equation

$$CO_2 + 4\,HO^*H \rightarrow CH_2O + 2\,H_2O^* + H_2O + O^*_2,$$

(where the marked atoms presumably indicate the initial origin of the oxygen). Bacterial photosyntheses, e.g., of the type

$$CO_2 + 2\,H_2S \rightarrow CH_2O + H_2O + 2\,S,$$

may indeed require no photolysis of water as such. No definitive experiments with the Hill reaction have been performed with these bacteria; corresponding studies with Athiorhodaceae suggest that the H-donors which these organisms require reduce the [OH] produced by photolysis. Nevertheless a real difference may lie in the photolysis act itself, the subsequent H-pathway being identical.

PHOTOCHEMICAL ASPECTS

The initial act of photosynthesis must be absorption of a quantum of light at a wave length not exceeding the red maximum of the particular chlorophyll concerned. (The lower wave length limit is not known; presumably it is a matter of where destruction exceeds utility). As is now common knowledge, the primary absorber may be some non-chlorophyll pigment, such as the phycobilins, or, to some extent and to varying degrees of specificity, the carotenoids (68). Chlorophyll is not always the sole, or occasionally, even the initial molecule absorbing the light. For example, action spectra[2] of *Nitzchia* photosynthesis show that in the blue region, the amount of light absorbed by the chlorophylls is insufficient to account for all the photosynthesis obtained. By subtracting the fraction of the photosynthetic yield contributed by the chlorophylls from that actually found, a curve of the absorption spectrum of the contributing pigment is obtained. These curves are found to fit one or more of the carotenoids present, and since, in these cases, the

[2] Action spectra are curves in which the amount of the phenomenon, in this case photosynthesis, is graphed as a function of the wave length or frequency of the incident light responsible for the phenomenon.

carotenoids are the only pigments present in amounts sufficient to account for the quantity of light absorbed, some of the carotenoid family must then be active in photosynthesis (25, 26) in this (but not necessarily all) species. However, no examples of plants or bacteria are known where photosynthesis proceeds in the absence of chlorophyll, even though carotenoids are present. Consequently, it is inferred that the carotenoids are able to transfer their energy to chlorophyll in some manner. The mechanism of this transfer is still obscure. Furthermore, since all carotenoids do not possess this property, one may presume that the ability of a particular carotenoid to contribute to photosynthesis may be associated with its geometric relation to chlorophyll, so that only those in unique positions are able to transfer their energy (see Fig. 1 B).

At the other extreme of action spectra we have those plants where the chlorophyll absorbs light but does not contribute appreciably to photosynthesis. This is particularly true of the red algae, where it may be shown that the action spectrum corresponds to the light absorbed by the phycobilin pigments, phycoerythrin and phycocyanin (42). These bilins fluoresce, as does chlorophyll. It may be shown that illumination of the red algae with light which the bilins absorb appreciably, and which chlorophyll does not, nevertheless results in fluorescence by the chlorophyll-*a*, not by the bilins. This constitutes evidence (as will be shown below) that the bilins transfer their energy to the chlorophyll-*a* and that the latter, though photochemically inactive, is the pigment responsible for the liaison between the photochemical act and the biochemical processes.

When a chlorophyll molecule is struck by a photon, certain electrons absorb the energy by moving to an outer, less stable, orbit. We describe this electronic transition by saying that the molecule is in its first excited singlet state, using the notation $M \rightarrow M^s$. The light which is absorbed provides the usual " absorption spectrum ". The lifetime of this first excited singlet state is about 10^{-8} to 10^{-10} sec. (energy from a higher level of this excited state is dissipated as heat to the excited ground state level in about 10^{-12} sec.). Energy may be lost from this level in a number of ways: dissipation by heat to the ground state, engagement in a chemical reaction, emission of a quantum of light (fluorescence), transfer of energy to other ground state molecules of the same species (inductive resonance) or by radiationless conversion to a metastable state

(internal conversion) of still longer lifetime, the triplet state, M^T. The last is equivalent to the separation of a π-electron bond into its component electrons, since the triplet state has been shown to be paramagnetic. In the triplet state, liftime may be 10^{-3} to 10^{-4} sec. or longer in exceptional circumstances. Internal conversion is temperature dependent. Consequently the emission of a quantum of light from the triplet state (long lived fluorescence, or prosphorescence) (56) is best observed at low temperatures, where its probability exceeds the probability of thermal degradation.

It was apparent from the early measurements of chlorophyll concentration in leaves by Willstätter, that the excitation of any one chlorophyll molecule by an average flux of photons, would require upward of 20 to 30 minutes for the initial reduction of carbon dioxide by that same molecule (i.e., the time for any one molecule to receive a total of four quanta). Alternatively, the number of molecules which would be required to pool the successive quanta would be around 2–300. Since it is readily possible to obtain initial rates of photosynthesis without induction periods, some means of energy transfer from molecule to molecule must exist.

A number of theories now exist which describe mechanisms for the migration of excitation energy (13): *a*) the sensitized fluorescence (inductive resonance) theory; *b*) the exciton theory; *c*) the lattice conduction band theory; and *d*) the proton migration theory. For energy transfer to occur via inductive resonance, the fluorescence band of the excited molecule must overlap an absorption band of the (indirectly excited) acceptor molecule. The closer the correspondence of the two bands, the greater the efficiency of transfer, with a critical distance, R, according to Förster's (31, 32) calculations of

$$R = \frac{\lambda}{2\pi n}\left[\frac{3}{8}\frac{t}{t_s}\frac{1}{\Delta\mu}\right]^{\frac{1}{6}}$$

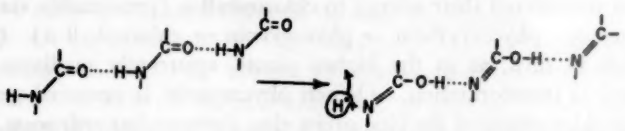
where λ is the mean wave length of the two bands in question, n is the refractive index of the solvent, t and t_s the respective actual and theoretical (unquenched) lifetimes of the excited state, and $\Delta\mu$ is a function of the overlap of the bands. For chlorophyll the "critical" molecular distance, that is, the distance separating an excited molecule from a non-excited one and at which the probability of transfer of excitation energy equals one-half, has been calculated

to be ~80 Å. Thus it has been shown that chlorophyll-*b*, whose fluorescence band overlaps the red absorption band of chlorophyll-*a*, sensitizes the latter at a concentration of 2×10^{-4} M (equivalent to one molecule each per 207 Å^3 or an average *b* to *a* distance of 103 Å^3). The theory of sensitized fluorescence is particularly useful for explaining the action spectrum of the red algae (42) where the phycobilins are the photon acceptor, the chlorophyll-*a* being virtually inactive. Fluorescence measurements show that the bilins have transferred their energy to chlorophyll-*a* (presumably via the sequence: phycoerythrin → phycocyanin → chlorophyll-*a*) (35) which in turn, as in the higher plants, apparently mediates the chemical transformation. (When phycocyanin is removed gently from chloroplasts of the blue-green alga *Synecocchus cedrorum*, the capacity for performing the Hill reaction is lost) (72). It appears rather more doubtful, however, that a similar explanation can be invoked for the sensitization of chlorophyll-*a* by the carotenoids, since there are conflicting claims concerning the fluorescence of carotenoids in the visible, and the writer is unaware of any studies on long-lived fluorescence or phosphorescence of carotenoids or their prototypes. The absence of these data may be, however, the result of very poor yields.

Of the remaining theories of energy transfer, neither the excitation theory nor the lattice conduction band theory, both of which are applicable to phenomena associated with crystal lattices, appears to have an experimental basis in photosynthesis. In the highly concentrated grana, well oriented chlorophyll molecules might well be analogous to a crystal lattice. For "exciton" transfer energy to occur, there must be strong interaction between the molecules, so that energy transfer will occur in a time short compared to that of the atomic oscillations within the molecules (26). The strong intermolecular interactions should be reflected in pronounced shifts in absorption spectra with increasing concentration. This does not appear to be the case.

In certain types of crystals, electrons which absorb photons may be excited into a conduction band, where they may move rapidly (leaving behind positively charged holes) until they are trapped at other holes or at an imperfection in the crystal. It has been suggested that the arrangement of peptide chains in a protein permit such electron transfer (30), although, as Duysens points out (1952,

ibid), these require electrons excited by a energy equivalent at least to that afforded by light of wave length 400 mμ (~ 72 kcal.). Since the electrons from chlorophyll-*a* have energies equivalent to ~ 40 kcal., at least two quanta would be required to initiate such a transfer. [A triplet state molecule, absorbing another photon to the excited triplet state (see Franck, 1955) would represent such a two-quantum molecule]. Finally it is not impossible that in the chloroplast protein a protein shift type of transfer:



may be involved in the location of photochemically produced "reducing power". Other hypotheses describe the adjacent peptide bonds of peptide chains as pseudo-conjugated systems, permitting movement of electrons with an ease approaching aromatic conjugated systems (see Bayliss, *loc. cit.*, for a summary of pertinent theories). Indeed, X-ray analysis of bond distances and angles suggest an enol contribution to the peptide bond of about 40% (61). To the extent that the peptide bonds of adjacent chains form a linear conducting system, the proton shift transfer may also be visualized as electron transfer.

The efficiency of energy transfer of the chlorophylls by exciton transfer or lattice conduction implies a high degree of orientation. Recent electron micrographs of the grana of (47) corn chloroplasts have been interpreted as in Fig. 1 A. The electron microscope itself is, however, not yet capable of determining orientation of molecules as small as those of chlorophyll.

The orientation of the pigments in the chloroplast may be studied by the measurement of dichroism and the degree of polarization of chlorophyll fluorescence. In measurements of dichroism, where one determines the birefringence as a function of the orientation of a molecular species, theory shows that there is an anomalous dispersion about any absorption band. This has been confirmed for chlorophyll-*a* dissolved in aluminum oleate and oriented by pressing the solution between glass plates (39, 40).

By variation of the angle of incidence, it may be shown that the

maximum absorption of light occurs when its plane of vibration is parallel to the porphyrin ring of the chlorophyll. Measurements of the anomalous dispersion of chlorophyll-*a* in aluminum oleate display residual birefringence at the mid-point of the curve, where theoretically none is to be expected. This residual birefringence is attributed to the orientation of the lipid molecules. In studies on

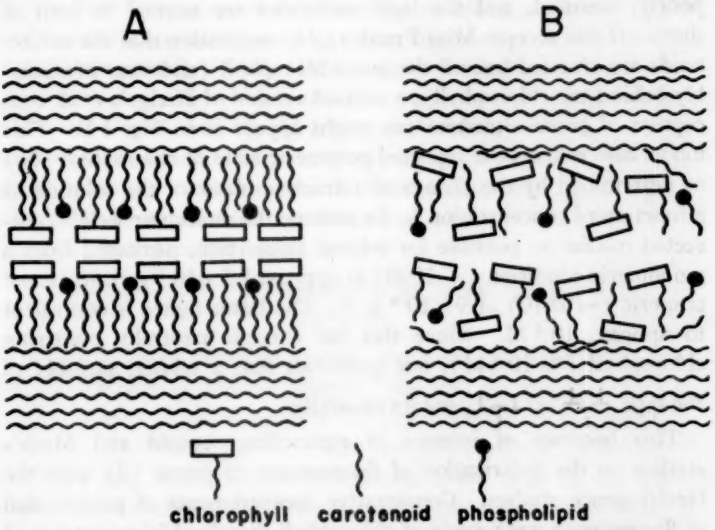


FIG. 1. A. Ultrastructure of the grana, as adapted from Hodge, McLean and Mercer (47). B. Revised ultrastructure, based on the evidence suggesting poor orientation of the porphyrin, apparent trimerization of chlorophylls and orientation of carotenoids parallel to porphyrins, with other lipids normal to the pigments and the general direction of the lamellae. The porphyrins are placed, in part, facing the protein, providing opportunity for electron conduction as well as Mg charge transfer.

chloroplasts of *Mougeotia* (Goedheer, *ibid.*) a third type of birefringence is also measured: the "form birefringence" resulting from micro-tissue orientations, e.g., lamellar structure, etc. By determining the residual birefringence at the mid-point of the anomalous birefringence (that is, at 680 mμ, the mid-point of the absorption band, where the contribution to the birefringence by the pigment is zero), the contribution of form birefringence and lipid orientation to the total birefringence is determined. This is pre-

sumed to be independent of wave length in the visible region. One may then calculate the expected contribution of pigment and deduce from the observed values the the degree of orientation of the chlorophyll. In this manner it has been shown that the porphyrin rings of the chlorophylls are oriented, though rather poorly, parallel to chloroplast lamellae. Carotenoids are similarly, though even more poorly, oriented, and the lipid molecules are normal to both of them. If one accepts Miss Frank's (34) suggestion that the carotenoids are situated behind the protochlorophyll (and thus presumably behind the chlorophyll), a revised version of Hodge's et al. conception of grana ultrastructure might appear as in Fig. 1 B. This model also reflects the reported polymeric state of chlorophyll (66) as determined by the change of refractive index of the solution as a function of concentration [3]. In acetone the molecular weight, corrected insofar as possible for solvent association, increased from a monomeric condition (~ 1000) at approx. 1.5×10^{-4} g./l. to almost trimeric (~ 2610) at 9×10^{-4} g./l. The latter figure is equivalent to approx. 10^{-6} M. Since this far exceeds naturally occurring chlorophyll ($> 10^{-2}$ M), one presumes that a trimer, possibly of the type ⊥⊤⊥ or ⊥⊤⊥, occurs naturally.

This becomes of interest in reconciling Arnold and Meek's studies on the polarization of fluorescence of grana (2) with the birefringence studies. Comparative measurements of polarization of fluorescence were made of chlorophyll dissolved in castor oil; of an aqueous chlorophyll protein preparation (presumably grana residue); and of *Chlorella*. Scattering was so profound in the last, that measurements could be made only in the region of 630 to 600 mμ. A very low value of polarization in the live plant was found, $\sim 20-25\%$ that of the chlorophyll-protein preparation, and $\sim 12\%$ that of the dissolved chlorophyll. The low intensity of

[3] Calculations were approximate, based on the equation $Sc/t = (1/M) + 2Be$, where M = molecular weight; B is a constant, dependent on the solvent; c is the concentration of the solute; t = "turbidity" = SMc, and

$$S = \frac{32\pi^3}{3} \frac{n_o^2}{N\lambda^4} \frac{n - n_o^2}{c},$$

where n = refractive index of the solvent, n_o = refractive index of the solution at the wave length used, λ = wave length used, N = Avogadro's number.

fluorescence in the live plant could imply a short lifetime of the excited state. Were this true, the polarization of the live plant should be greater than that of the chlorophyll in the castor oil.[4] Since this is not the case, molecular rotation is not of itself responsible for the depolarization. Another means of depolarization is that of energy transfer between randomly oriented molecules of a high enough concentration to permit energy transfer. Using values of the Smoluchouski-Einstein equation, higher values P were predicted than actually found. If, however, chlorophyll is trimeric in its natural state, then V is more than three times as large as used in the calculation, and P, which is correspondingly less, approaches experimental values. From the values of P obtained, it is calculated that the excitation rate of transfer from one chlorophyll to another is 15 to 20 times as fast as the energy is used for photosynthesis or heat degradation.

TRANSFER OF PHOTOCHEMICAL TO BIOCHEMICAL ENERGY

As noted in the previous section, there are plausible theories for energy transfer in photosynthesis. As will be shown later, the major aspects of photosynthetic carbon assimilation are probably solved. The mechanism by which energy is transferred from the photochemical to the biochemical system is the crux of the photosynthesis problem and the area where speculation is most prominent.

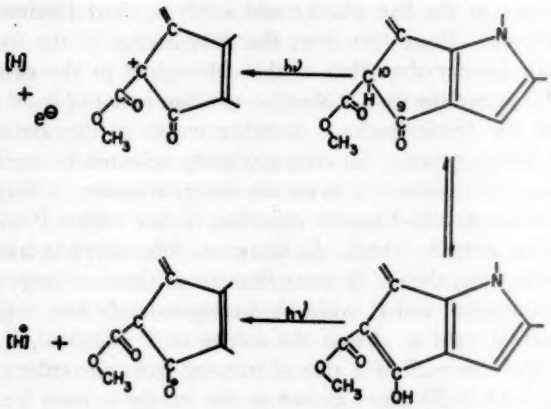
Synthetic chlorophyll-protein systems capable of photochemical quinone reduction have not yet been devised. Consequently it is not known whether chlorophyll itself serves as the direct H-donor, or whether an intermediate is necessary.

Numerous examples of the direct action of chlorophyll have been suggested. For example one may speculate that chlorophyll is ionized or dissociated via reactions about the H of C_{10}:

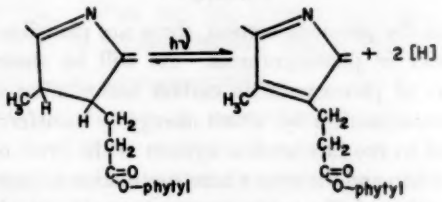
[4] Calculations were based on the Smoluchowski-Einstein equation:

$$\frac{1}{P} - \frac{1}{3} = \frac{1}{P_o} - \frac{1}{3}\,1 + \frac{RT}{n^v}t,$$

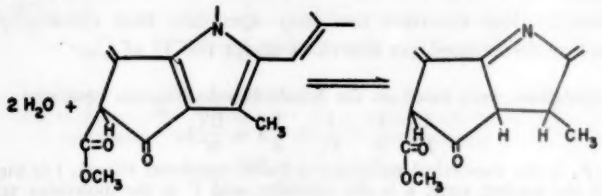
where P_o is the theoretical polarization (with rotational effect), t is the lifetime of the excited state, n is the viscosity, and V is the molecular volume of the pigment. If $t_{\text{plant chlorophyll}} < t_{\text{solution chlorophyll}}$, then, according to the above equation $P_{\text{live plant}} > P_{\text{solution}}$.



Secondly, the two hydrogens of ring IV may be involved in a chlorophyll–protochlorophyll redox system:



but exploratory experiments with D_2O (21) showed no exchange. Still a third type of direct H-transfer maybe imagined, namely, a chlorophyll → bacteriochlorophyll reaction of the type:



where one would expect a diminution of the red band and the appearance of a band in the infra-red. Experiments testing this conjecture have not yet been performed.

A third theory (54) involves the univalent oxidation of the magnesium in the chlorophyll molecule:

$$\text{pheophytin} < \text{Mg} \xrightarrow{h\nu} \text{pheophytin} < \text{Mg}^*$$

$$\xrightarrow{R} \text{pheophytin} < \text{Mg}^+ + R^-$$

$$2\,\text{pheophytin} < \text{Mg}^+ + 2\,\text{HOH} \rightarrow 2\,\text{H}^+ + \tfrac{1}{2}\,O_2 + H_2O.$$

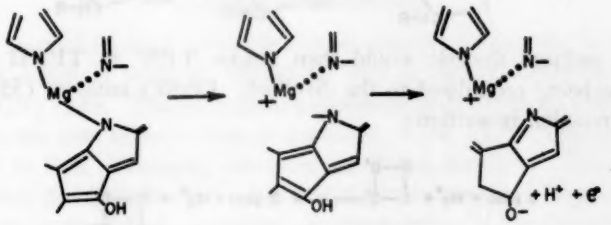
The direct photo-ejection of an electron: pheophytin $< Mg^* \xrightarrow{h\nu}$ pheophytin $< Mg^+ + e^-$ is calculated, on the basis of $Mg^\circ \rightarrow Mg^+$, to require a potential of 7.61 mv which is equivalent to light of wave length 162 mμ and consequently unavailable by solar photosynthesis. There appears to be a correspondence of the Mg° and Mg^* emission band with chlorophyll absorption and fluorescence bands as well as a lack of correspondence with other elements of the same family or period. That a porphyrin must be chelated, e.g., with Mg or Zn, to participate in photochemical reductions, was demonstrated by Dorough and Calvin's studies (23) on the photochemical conversion of α,β,γ,δ-tetraphenyl chlorin to the corresponding porphyrin. Furthermore, Livingston has recently shown that the base-activated fluorescence of chlorophyll in non-polar solvents must be associated with the Mg, since pheophytin does not fluoresce under similar conditions.

In discussing this reaction, Marcus (59) emphasized the stabilization effect of the solvent on the otherwise very transient univalent ion, suggesting the generalized kinetics:

$$\text{pheo} < \text{Mg} \cdot H_2A \rightleftarrows \text{pheo} < \text{Mg} \cdot H_2A^- \rightarrow \text{pheo} < \text{Mg}^+ \cdot H_2A + [\text{H}]$$

$$\text{pheo} < \text{Mg}^+ \cdot H_2A + H_2O \rightarrow \text{pheo} < \text{Mg} \cdot H_2A + \tfrac{1}{2}\,O_2 + \text{H}^+$$

Alternatively, the reaction may be depicted as:



Because of the correspondence of most of the principal absorption lines of the chlorophylls to Mg^+ as compared to Mg°, Levitt suggests a high probability of the reaction

$$\text{pheo} < Mg^+ \xrightarrow{h\nu} \text{pheo} < Mg^{+*} \xrightarrow{R} \text{pheo} < Mg^{++} + R^-$$

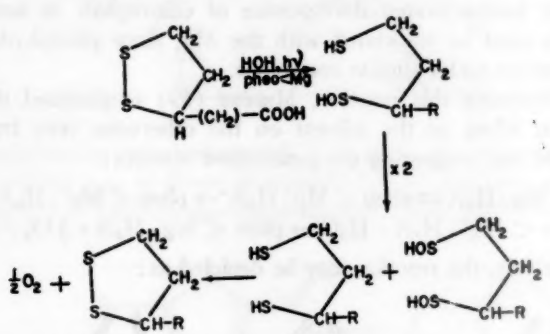
The statistical probability of such a reaction must be rather small, unless the geometry of the grana is such that all photons are trapped at a particular pigment locus. However, it is interesting to consider the plausibility of the "electronic dismutation"

$$\text{pheo} < Mg^+ + \text{pheo} < Mg^* \rightarrow \text{pheo} < Mg + \text{pheo} < Mg^{++} + e^-.$$

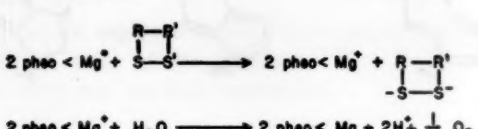
The doubly-ionized molecule would then react with water to regenerate chlorophyll:

$$\text{pheo} < Mg^{++} + 2\,HOH \rightarrow \text{pheo} < Mg + \tfrac{1}{2}\,O_2 + 2\,H^+ + H_2O.$$

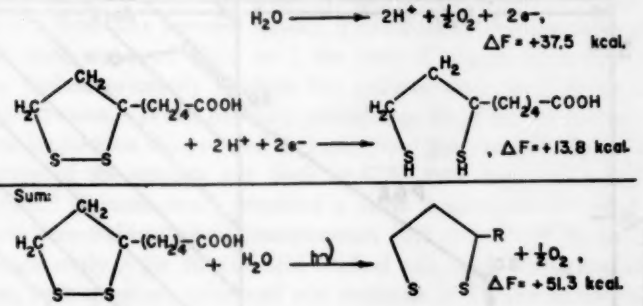
While the "solvent stabilizing" group written above as H_2A may be H_2O, it may also be other substances, as protein. Indeed the 2 H's of H_2A are superfluous, since any electron acceptor would be adequate. Thus Calvin and colleagues have suggested that thioctic acid may play such a role (22):



The reduced thioctic would then reduce TPN^+ to $TPNH + H^+$ while being reoxidized to the disulfied. Levitt's analysis (55) of this reaction is written:

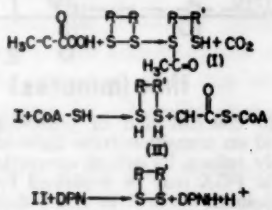


A thioctic acid model (trimethylenedisulfide) has been shown by Calvin and his co-workers (9) to participate in photo-oxidation by zinc tetraphenylporphine, one atom of oxygen being absorbed per mole of disulfide. The simplified thermodynamics of the photolysis of water has been represented as the sum of two reactions:



Other calculations show values ranging up to $\cong 65$ kcal. Since light from two quanta at 700 mμ is equivalent to 81.4 kcal., the former calculation would indicate photolytic transfer efficiency of 51.3/81.4 = 0.63.

Thioctic acid has been particularly attractive because of its known role in the decarboxylation of pyruvic acid to "active acetate" which is then transferred to CoA:



the acetyl CoA then being condensed with oxalacetic acid to citric acid which is oxidized further by way of the tricarboxylic acid (TCA) cycle. Photosynthetic experiment had shown (see Fig. 2) that the rate of formation of members of the TCA cycle is very low in light, increasing relatively rapidly in the dark. This suggested that in light the thioctic acid might be kept to a large extent in the reduced form, inhibiting decarboxylation of the pyruvic acid and, in turn, the operation of the TCA cycle. (Actually the in-

crease in TCA components in the dark is roughly equivalent to the diminution in malic acid. An alternative explanation thus consists of photosynthetic malic acid entering into the TCA cycle when dissociated from its photo-enzyme system in the dark. More recent data (12b) report no change in rate on passage from light

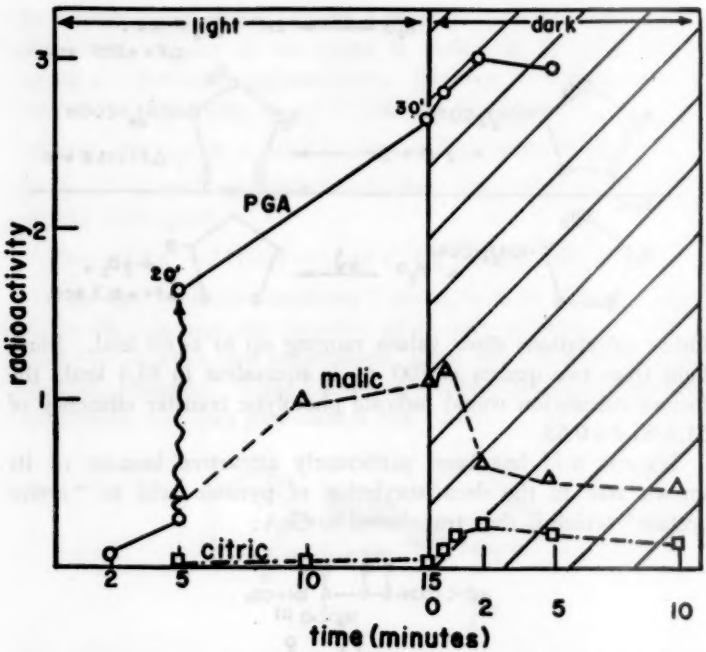


FIG. 2. The change in concentration of phosphoglyceric acid (PGA), malic acid and citric acid on transition from light into darkness. The increase in PGA presumably reflects its lack of conversion to triose phosphates. The subsequent decline in PGA may be explained by its transformation to alanine and related compounds. It will be noted that the increase in citric acid may be accounted for by the entrance of malic acid into the tricarboxylic acid cycle. (Adapted from Calvin, M., UCRL 2924, March 1955.)

to darkness, or vice versa; instead there is continual increase. Assuming that malic acid is a bona fide member of the TCA cycle, it is difficult to reconcile its steady increase with the dark-light concentration dependency of the other members of the cycle.).

Only about one-third of the total thioctic acid is found in the chloroplast (37). Addition of thioctic acid to a quinone Hill re-

action results in an increased rate of the reaction when the quinone is rate-limiting (17, 18). These reactions, as the authors point out, provide no evidence concerning the photochemical role of thioctic acid, since the latter may be merely controlling a dark reaction. When the reaction was performed in flashing light (Bradley and Calvin, 1955), using entire algae (*Scenedesmus*) and varying the dark time between flashes, it could be shown that when the flash time was brief (0.05 sec.) the yield of oxygen from the flash was limited primarily by both the quinone and the thioctic acid concentration. When the dark period was lengthened (0.20 sec.), these limitations disappeared and only the light was limiting. Estimation of the oxygen per flash at 0.20 dark time (2.94×10^{-12} M/mm.^{3} *Scenedesmus*) provided a value approximately equal to the *in vivo* 6-thioctic acid concentration ($3.8 \pm 1 \times 10^{-12}$ M/mm.^{3}). Consequently if the thioctic acid worked only once during the dark time, each quantum produced one molecule of O_2 per molecule of thioctic acid—which cannot be the case, since four electrons are required per molecule of oxygen. Consequently it was suggested that the thioctic acid performs a number of reactions during the dark period, and, as a corollary, that there is a reservoir of stored photochemical energy available to the thioctic acid during this period—possibly an extra-long-lived state of chlorophyll. The minimum lifetime of this extra-long-lived state was calculated from the data for yield saturation under the varying conditions. The longest time (0.23 sec.) was found for the $0.5 \times$ quinone addition, the shortest (0.06 sec.) with added thioctic acid. The latter finding is the crux of the argument, since this value is reasonably near the earlier data reported for *Chlorella* photosynthesis (0.02 sec.) and the Hill reaction (0.04 sec.). *Scenedesmus* appears to be unique in the inhibition of the Hill reaction by excessive quinone. At 0.003 M quinone the minimal dark-time for flash saturation is only 0.13 sec., but even this value is reduced to 0.06 sec. by addition of the 7×10^{-4} M 6-thioctic acid. The 6-thioctic acid therefore serves as a more efficient H-acceptor than quinone and is presumably closer to chlorophyll.

Livingston's studies have suggested a lifetime of 10^{-3} to 10^{-4} sec. for triplet state chlorophyll (56). Witt (85) has observed spectral changes on illumination of algae which are ascribed to triplet state chlorophyll and have a life time of about 0.02 sec.

Since this latter time is still too short to explain the 0.23-sec. flash saturation period, Bradley and Calvin believe an extra-long-lived state of excited chlorophyll exists. The thioctic acid reacts only with the products of this second transformation. Their general scheme is as follows:

Step 1. pheo < Mg → pheo < Mg^a ($t \sim 10^{-8}$ sec.)
Step 2. pheo < Mg^a → (metastable-state) pheo < Mg^t ($t \sim 0.03 - 0.06$ sec.)
Step 3. pheo < Mg^t → pheo < Mg + heat
or Step 4. pheo < Mg^t → pheo < Mg + luminescence
or Step 5. pheo < Mg^t → pheo < $Mg^+ + e^-$
Step 6. pheo < $Mg^+ + HOH$ → pheo < $Mg + \frac{1}{2} O_2 + H^+$

Step 7. $e^- + \frac{1}{2}$ (S—S) $+ H^+ \longrightarrow \frac{1}{2}$ (SH SH)
persulfide (thioctic) dithiol (reduced thioctic)

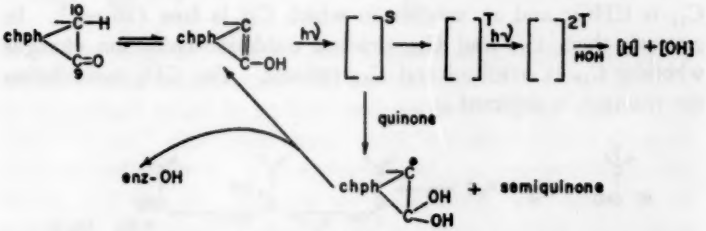
Step 8. —(HS SH) + { $\frac{1}{2}$ benzoquinone → hydroquinone ; $\frac{1}{2}$ TPN^+ → $\frac{1}{2}$ TPNH + H^+ } + $\frac{1}{2}$ (S—S)
dithiol (reduced thioctic) persulfide (thioctic)

The authors are inclined to ascribe the positive charge to the "hole" left by the removal of an electron from the "conduction band" formed by the array of chlorophyll, rather than the unipositive pheo < Mg^+ proposed by Levitt.

On the basis of similar studies with flashing light, Allen and Frank (1) have also suggested that some photoproducts may have a lifetime considerably longer than the 10^{-2} sec. found by Emerson and Arnold. This is based upon the observation that in quinone-treated *Scenedesmus* the combined yield of two short (saturating) flashes of light increases as the time interval between the flashes decreases. Thus a 30% increase is obtained when the flashes are 1 sec. apart as compared to 250 sec. apart. Consequently it is suggested that the first flash produces something which requires a second flash for oxygen production, something whose rate of conversion to oxygen is accelerated by the second flash. It was observed that a single short (5×10^{-4} sec.) flash evolved oxygen in the Hill (quinone) reaction, but not in the photosynthetic (CO_2) reaction. When a 50 times as long (but lower intensity) flash was used or when the system was "primed" with continuous very low

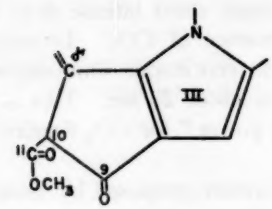
level illumination, a single short intense flash resulted in oxygen production in the presence of CO_2. Interestingly enough, the priming resulting from continuous illumination persisted in the dark with a half life of about 20 sec. This is reminiscent of the survival of " reducing power " for CO_2 fixation in pre-illuminated *Chlorella.*

The excitation mechanism proposed by these authors is as follows (33):

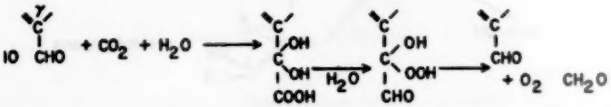


The scheme revolves about carbons 9 and 10 of chlorophyll. The first excited singlet state possesses sufficient energy to effect a reduction of quinone to the semiquinone (but not enough to dismute water). The chlorophyll-free radical thus formed is presumed to react oxidatively with an enzyme, restoring the chlorophyll (enolate). To dismute the water the energy of a second quantum is required. It is suggested that stabilization of the first triplet state by complex formation results in a high probability of its being activated to the second triplet state (2 T) (that is, an excited triplet). This 2 T molecule then has sufficient energy to dismute water. The change in yield with varying times between flashes in the quinone-treated *Scenedesmus* is consequently explained as a competition between the relatively shorter-lived one-quantum process and the two-quantum process. If the time interval is short, the one-quantum process is heavily populated and more oxygen is obtained than with a longer time interval where some loss has occurred in the time required to populate the 2 T species.

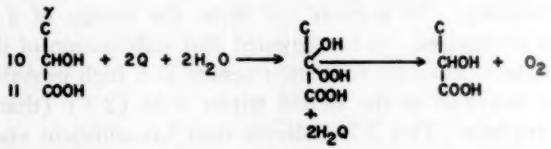
Warburg and Krippahl (82) have revived the original Willstätter theory of direct participation of chlorophyll in photosynthesis. They assume three kinds of chlorophylls in addition to the common, highly predominant one: *a*) a chlorophyll in which C_{11} is present as a carboxyl group which may take part



in phosphorylations and acylations; *b*) a chlorophyll-*a* in which C_{11} is CHO; and *c*) another in which C_{11} is free (absent). In general, then, C_{10} and C_{11} conduct oxidation-reduction changes whereby C_{10} is oxidized and C_{11} reduced. For CO_2 assimilation the reaction is depicted as:



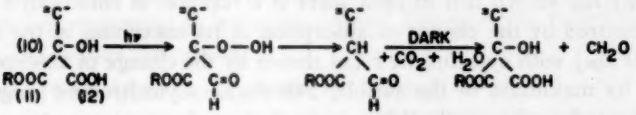
The energy for the scission shown in the last step is presumed to arise by the coupled oxidation of —CHO to —COOH in other illuminated chlorophylls. The reduction of quinone is pictured as fortuitous, having nothing in common with CO_2 assimilation:



That C_{10} of chlorophyll may be the hub of the photochemical function has been recognized by many workers in this field (see, e.g., the extensive discussion by Rabinowitch) (63). That chlorophyll is involved directly in CO_2 assimilation ignores all of the isotopic work of recent years on CO_2 fixation, which has culminated (see below) in the isolation of a colorless enzyme which couples CO_2 with ribulose diphosphate to produce phosphoglyceric acid. Indeed, thermodynamic calculations (see below) suggest that this carboxylation may proceed spontaneously (i.e., $\Delta F \cong -9$ kcal.). The designation of formaldehyde as a product of the reaction may be presumed symbolic—since here, too, it has beer shown (38)

that the feeding of trace (non-inhibitory) amounts of formaldehyde-C^{14} does not result in rapid assimilation into the usual products of photosynthesis.

A subsequent publication (82a) proposes yet a different model for direct chlorophyll participation in CO_2 assimilation:



It is then suggested that the additional carboxyl group is of such lability that all known techniques for the extraction and purification of chlorophyll result in its loss, and the proof of the existence of such "native" chlorophyll must await the development of novel, more refined methods. The asymmetry of C_{10} confers individuality on C_{11} and C_{12}, i.e., they are not equivalent. Consequently one would not necessarily expect $C^{14}O_2$ to result in rapid labeling of chlorophyll in C_{11} as one would if these were equivalent and the rearrangement of C_{10} to a peroxide were compatible with either C_{11} or C_{12}. The major basis for the above mechanism is the quantitative equivalence of the chlorophyll concentration and CO_2 produced by the addition of 4×10^{-3} fluoride to *Chlorella*. Obviously such parallelism in concentration must follow for every system related to the photosynthetic system. Thus, if the working level of phosphopentose carboxylase is equal to that of chlorophyll, and if the CO_2-complex of this enzyme is dissociated by fluoride, the amount of CO_2 released will be equivalent to the chlorophyll concentration. Consequently the proposal for direct participation of chlorophyll in CO_2 reduction is based upon very indirect evidence. There is at present no means of verifying this theory. Almost all investigators now believe that chlorophyll itself does not participate directly in carbon dioxide assimilation.

As the average number of the photons per unit time striking a chlorophyll assemblage is very small compared to the number of chlorophyll molecules (a ratio of about 1/200 at most), changes in the chlorophyll molecule resulting from photochemical transfer, and which may be observed spectrophotometrically[5], are corre-

[5] That is, one observes the solution with a small reference beam, insufficient to produce appreciable photochemical reaction. This establishes the

spondingly small. That chlorophyll itself may take part in photochemical oxidation-reduction reactions, usually involving the bleaching of the chlorophyll, has been known for some time (63), various model systems being employed, e.g., chlorophyll with Fe^{+3}, with ascorbic acid, with riboflavin, etc. For example, Krasnovskii (52) has shown that in light there is a reaction of chlorophyll (as measured by the change of absorption at its maximum in the red, 670 mμ) with cytochrome *c* (as shown by the change of absorption at its maximum in the visible, 548 mμ). Cytochrome-*c* may be acting photochemically either as an electron donor or acceptor. An electron acceptor mechanism would then be written as:

$$\text{Chl} + \text{AH} \xrightarrow{h\nu} \text{Chl}^- + \text{AH}^+$$
$$\text{Chl}^- + \text{cyt. Fe}^{+3} \rightarrow \text{Chl} + \text{cyt. Fe}^{+2}$$
$$\text{AH}^+ \rightleftarrows \text{A} + \text{H}^+, \text{ etc.}$$

while the electron donor mechanism could be formulated as

$$\text{Chl} + \text{cyt. Fe}^{+2} \xrightarrow{h\nu} \text{Chl}^- + \text{cyt. Fe}^{+3}$$
$$\text{Chl}^- + \text{O}_2 \longrightarrow \text{XO}_2^- \xrightarrow{\text{H}^+} \begin{cases} \text{irreversibly oxidized ch.} \\ \text{regeneration of Chl e.g.} \\ \text{Chl} + \text{HO}_2, \text{ ect} \end{cases}$$

Duysens, working with whole plants (27–29), had earlier found changes in the absorption spectrum upon illumination, which were interpreted as the result of electron transfer in the cytochromes and the pyridine nucleotides. With *Chlorella,* an actinic beam of $\lambda > 670$ mμ showed an increase at 515 to 520 mμ and decreases at about 478 and 420 mμ, similar effects being found in a leaf, a sea algal thallus and in the blade of *Vallisneria.* The change at 420 mμ was believed to be associated with changes in the spectrum of cytochrome *f,* which is unique to plants and is believed to have a role as an electron carrier in photosynthesis (24, 45). The initial suggestion was confirmed by studies of the difference spectrum of the red alga *Porphyridium,* which displayed a strong negative change at 420 mμ and a smaller one at 555 mμ, both bands corresponding to cytochrome *f.* With this organism the bands at 480

amount of chlorophyll. The actinic beam impinges normal to the reference beam. The latter is then utilized to observe the actinic changes (due account being made for fluorescence and scattering).

and 520 mμ were so small as to be lost. Similarly the difference spectra (29a) for *Rhodospirillum rubrum* and *Chromatium* show diminutions of the bacteriochlorophyll bands at 880 and 810 mμ, judged to arise most probably from two separate pigments. Simultaneously there were increases at 790 mμ and 430 mμ. These are ascribed to oxidation of bacteriochlorophyll to an unknown compound, suggested as bacterioviridine. The sequence of electron transfers is then written as:

Bact. Chl.* + X → oxidized bact. chl. (bacterioviridine)
+ XH (photosynthetic reductant)
oxid. bact. chl. + red. cytochrome + proton(s)
→ bact. chl. + ox. cyt.

An alternative explanation is the formation of bacteriopheophytin, resulting from photochemical displacement or ionization of the Mg, as discussed earlier.

Hill and Whittingham list the major plant cytochromes as *f* (characteristic only of chlorophyll-containing tissue), a_3, *b*, b_3 and *c*, which appear to be scattered throughout the plant, although highest in meristematic tissue (46). Lundegårdh (57) has indeed found that cytochrome *f* is mostly in the oxidized form in the light and in the reduced form in the dark, while cytochromes *b* and *c* appear not to be affected by light.

A considerable elucidation of the photosynthetic bacterial cytochromes has resulted from the work of Kamen and Vernon (49, 75–77). Thus from *Rhodospirillum rubrum* a cytochrome *c* similar to plant cytochrome *f* has been prepared. (It was called "bacterial cytochrome *c*" because of similar porphyrin moieties, that is, similar pyridine and cyanide hemochromogens—but differed from conventional cytochrome *c* in electrophoretic, adsorptive and enzymic properties). All photosynthetic bacteria thus far investigated—from the strictly autotrophic anaerobic (e.g., *Chlorobium*) to the anaerobic heterotrophs (as *Chromatium*) appear to contain this bactocytochrome *c*. Indeed, the non-photosynthetic autotrophic N_2-fixer, *Azotobacter vinelandii*, also contains this cytochrome, and, since it also contains the Ru-1,5-P carboxylase (36), a possible mutagenic loss of photosynthesis during its evolution is suggested. It is not present in the fermentative anaerobic *Clostridium pasteuranium*.

Bactocytochrome *c* is given a role both in light and dark hydrogen transfer analogous to that for cytochrome *f* in the higher plants. However, *R. rubrum* possesses a strong inhibitor of oxygen-uptake in light, which does not occur in the higher plants. Thus, if cytochrome oxidase is inhibited by cyanide, bactocytochrome *c* will oxidize DPNH photochemically. Consequently it is suggested that bacterial photosyntheses, in which hydrogen donors other than water are required for growth, photooxidize that hydrogen donor instead of water in accord with the lower potential requirements. Thus ". . . an intermediate in the photochemical hydrogen transfer is coupled through (bacto) cytochrome *c* to the oxidation of the hydrogen donor ".

REDOX POTENTIALS AND THE HILL REACTION

An elementary analysis of the empirical equation of photosynthesis—$CO_2 + H_2O \rightarrow CH_2O + O_2$—allows the latter to be written as the sum of two half reactions:

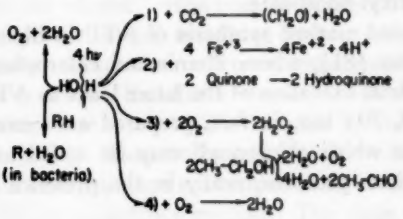
a)	$2\,H_2O \rightarrow 4\,H^+ + 4\,e^- + O_2$	$E'_o = -0.81$ v.
b)	$H_2O + CH_2O \rightarrow 4\,H^+ + 4\,e^- + CO_2$	$E'_o = +0.44$ v.
	$CO_2 + H_2O \rightarrow CH_2O + O_2$	$E'_o = -1.25$ v.

where the E'_o's refer to the potentials at pH = 7. The E'_o of the combined half reactions may then be converted to $\Delta F'_o$ by the relation $\Delta F'_o = (-E'_o)(n)(23{,}066)$, where *n* is the number of electrons involved and 23,066 is the defined volt equivalent. Consequently $\Delta F'_o = (-1.25)(4)(23{,}066) = 115{,}000$ cal. Reaction *a* states that any couple with a more negative potential than this will decompose water with evolution of oxygen, $E_o = -1.23$ v. Reference to Latimer's standard text (53) will reveal the existence of many such couples (e.g., $Ce^{+4} \rightarrow Ce^{+3}$, $Mn^{+3} \rightarrow Mn^{+2}$, etc.). These decompositions of water will, of course, proceed in the dark. The ferroferricyanide couple [$Fe(CN_6)^{-4} \rightarrow Fe(CN_6)^{-3} + e^-$, $E_o = -0.36$ v.] is then not sufficient to effect the scission of water, nor is that of hydroquinone–quinone [$E_o = -0.68$, $E'_o = -0.28$] (although the E_o of the first of the two electron-transfer reactions: quinone to semiquinone, semiquinone to hydroquinones, has an $E_o = -0.75$ v.). If, however, one were to pass a current of electricity through an aqueous solution of the ferricyanide with a potential equal to the difference between the water couple and the ferricyanide couple

[$\Delta E = (-)1.23 - (-)0.36 = 0.87$ v.], decomposition of the water would ensue (neglecting overvoltage effects). R. Hill first demonstrated (43, 44) that when chloroplasts were placed in the water, along with the ferricyanide, the decomposition of the water would occur if light instead of electricity were used. This phenomenon is now known as the Hill reaction and has been shown to occur with a variety of substances, for example, 2,6-dichlorophenolindophenol and quinone. (A list of the various pathways which the products of water photolysis may pursue is provided in Table I). It is obvious that when relatively less energy has to be put in to equate the water with its oxidant, the reaction will proceed with relative ease. In photosynthesis, where the oxidant is CO_2, the E'_o, as mentioned

TABLE I

GENERAL PATHWAYS OF PRODUCTS OF H_2O PHOTOLYSIS



photosynthesis

Hill reactions

Mehler-Hill reaction (not shown by living cells)

(endogenous catalase)

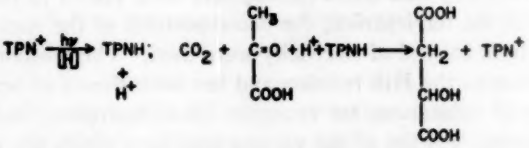
(xs catalase)

catechol oxidase, or cytochrome system

Adapted from A. H. Brown and N. Good. Photochemical reduction of O_2 in chloroplast preparations and in green cells. I. The study of O exchanges *in vitro* and *in vivo*. Arch. Biochem. Biophys. **57**: 340–354. 1955.

above, is +0.44. The apparent question then is, using oxidants other than CO_2, how much potential can be generated by the Hill reaction? The oxidant of lowest potential giving a Hill reaction appears to be potassium anthraquinone-β-sulfonate, with $E_o = -0.21$ v. ($E'_o = +0.21$ v.) (6). Consequently chloroplast preparations have not yet been prepared with a reducing potential sufficient for CO_2 reduction—but this may be merely the result of crudity of technique in chloroplast preparation. Macdowall (58) has obtained a potential drop of 0.687 v. with toluylene blue. Although this compound has an E'_o of −0.115, corresponding to an initial E_h of −0.448, the E_h found after 2.75 hrs. of Hill reaction was +0.239, corresponding to a total potential drop of 0.687 volts. TPN^+, which has a potential of +0.32 v., may be reduced photochemically by

chloroplasts (80), as a result of which it may be coupled with malic and isocitric enzyme to effect a net carbon dioxide fixation:



(and a similar reaction may be written for the reaction CO_2 + α-ketoglutaric → isocitric acid). Although the redox level of TPNH is not sufficient to reduce CO_2, it is sufficient to reduce a β-phosphorylated carboxylic acid (e.g., PGA ⇄ triosephosphate). Consequently, if a portion of the photosynthate is reoxidized to generate phosphate ester bonds, the photochemically produced TPNH may be used to reduce the carboxyl phosphate.

Strehler (69) has reported marked synthesis of ATP in illuminated chloroplasts (see also [81], where illuminated chloroplasts reduce DPN^+. Mitochondrial oxidation of the latter leads to ATP synthesis). Vishniac (78, 79) has, in fact, prepared an acetone powder of chloroplasts, to which chlorophyll may be added and which will reduce glutathione photochemically in the presence of TPN^+.

The most direct evidence for (anaerobic) photosynthetic phosphorylation (as well as actual CO_2 assimilation) has been found by Arnon and his colleagues (3, 5), using chloroplasts or, more recently, plasmoptized spinach chloroplasts. Similar photosynthetic activity has been found for fragments of *Spirogyra* (73). In Arnon's studies, illumination of chloroplasts in $P^{32}O_4^{-3}$ resulted in presumed [6] ATP^{32} or G-6-P^{32}, the product of the hexokinose reaction: G + ATP^{32} → G-6-P^{32} + ADP. The rate of phosphorylation is increased by the addition of indigenous chloroplast compounds, such as ascorbic acid and vit. K_1 (the most active preparations also required flavin mononucleotide (FMN) and Mg^{+2}). The additions of DPN, TPN, UTP, cocarboxylase, 6,8-thioctic acid, or a mixture of these did not increase the yield of photophosphorylation. Brad-

[6] That is, organic P^{32} (P_i^{32}) was absorbed on Norite (charcoal). Neither P_i^{32} nor sugar phosphates are thus absorbed. Further, acid liberated P_i^{32} indicating P_o^{32} esterification. However, other nucleotides are also absorbed. The better evidence, then, is the hexokinase reaction, which is specific for ATP.

ley (16) has pre-fed P^{32} to *Scenedesmus* (i.e., established stationary isotopic states) and chromatographed the light-dark transients in the presence and absence of CO_2 and oxygen. The changes in P^{32} thus presumably corresponded with concentration changes of the chromatographically separated compounds. The six or seven compounds reported (PGA, ADP, ATP, Ru-1,5-P, G-P, P_i and PPA) represent together about three-fourths of the total P^{32}. In agreement with the proposed mechanism for CO_2 assimilation, Ru-1,5-P was negligible in the presence of CO_2, while PGA was three to four times as high with CO_2 as in its absence. Profound changes in P_i were ascribed to technical difficulties associated with the decreased rate of assimilation of P_i in the dark compared to that in light and the combined analysis of P_i^{32} in the medium as well as that in the cells. Changes in the ATP, ADP levels were apparent, but not violent. However, it is argued that these are so close to the photochemical act, being both produced and used rapidly, that drastic changes are not to be expected. The data explain the experiments of Kandler (50), in which relatively small changes in steady state levels of TCA-soluble P_i were observed, falling $\sim 20\%$ on 30″ of illumination and rising $\sim 30\%$ after 90″ of darkness. The changes were not so profound as those suggested by Strehler (70) by an indirect method. The signs of the transients, however, were in agreement with the model of light-induced reduction of

$$TPN^+ \xrightarrow[H_2O]{[H]} TPNH + H^+ + \tfrac{1}{2}O_2,$$

oxidation of the reduced $TPNH_2$ being coupled with the phosphorylation of ADP:

$$H^+ + 3\,P_i + 3\,ADP + TPNH_2 + \tfrac{1}{2}O_2 \rightarrow TPN^+ + H_2O + 3\,ATP.$$

The rapidity of the rise of the PGA in the dark-to-light transition was interpreted as photophosphorylation of Ru-5-P to Ru-1,5-P, mediated by the ATP formed photochemically.

As a consequence of the beneficial effects in photosynthetic CO_2 fixation by the chloroplast-indigenous vit. K and ascorbate, Arnon and his colleagues have proposed an electron transport scheme of the following general outline:

$$\xrightarrow{[H]} FMN \rightarrow \text{Vit. K} \rightarrow \text{ascorbate} \rightarrow \text{cytochromes}$$

[That vit. K and ascorbate may be involved in photosynthesis in

this manner was apparently first suggested by Aronoff (7) and later, independently, by Wessels (83).]

ASSIMILATION OF CO_2

The five-minute assimilation of $C^{14}O_2$ has been studied with 27 plants representing nine phyla, covering the entire range of the plant kingdom (60). Paper chromatographic analysis of the products showed many qualitative similarities. Thus hexose monophosphates appeared to be universal, and pentose and triose phosphates were almost so, as were phosphoglyceric acid. The sugar-transferring nucleotide, uridine diphosphate glucose (UDPG), was generally present, as were ribulose diphosphate, phosphoglycolic acid and phosphoenolpyruvic acid. Serine and alanine were universal, while aspartic, glutamic and alanine were also generally present. The article suggests, however, that sucrose provides a means of dividing the plants into two groups, one in which the sucrose is less than 10% of the soluble radioactivity and another where it is more than 35% (at the end of the five minutes). The high and low sucrose levels are associated, respectively, with the presence or absence of non-photosynthetic tissue. The additional 2–5 Kcal. energy of the disaccharide, as compared to the monosaccharides, is the somewhat teleological argument adduced for this state of affairs. A rate of translocation of sucrose greater than the monosaccharides (74) is an equally satisfactory explanation. In support of this argument, mature moss and fern were shown to produce 48 and 37% sucrose, respectively, while the alternate, completely photosynthetic generation, moss protonemata and fern prothalli, produced only 23 and 24%, respectively. All in all, however, the similarity of compounds found suggested that the general photosynthetic pattern was similar, if not identical, in all green plants.

The establishment of a pattern, however, requires kinetic data. Thus, in the sequence $A \rightarrow B \rightarrow C \rightarrow$, where A, B and C represent concentrations of initial and subsequent products of photosynthesis, the initial slope of A will be linear, and its entire curve will be monotonic. The initial slope of B will approach zero, the overall curve being S-shaped. C will have an initial slope even less than that of B, etc. Fortunately the same laboratory (to which we are indebted for by far the major part of our knowledge concerning

intermediates in the assimilation of CO_2) has endeavored to obtain such kinetic data with algae (12, 14, 84). As the time of photosynthesis is diminished, the first observable product of photosynthesis with $C^{14}O_2$ is phosphoglyceric acid (PGA) (free glyceric acid is observed first in the soybean plant [8]), although the data for both phosphopyruvic and malic acids could be interpreted as showing finite slopes at the origin. The sugar phosphates (ribulose

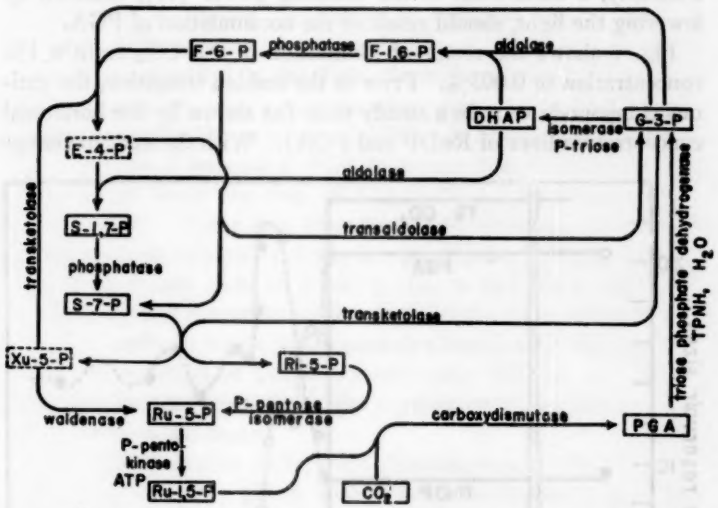
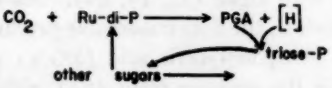


FIG. 3. The photosynthetic cycle. It will be noted that, according to this scheme, all reactions require erythrose-4-phosphate, which has not yet been identified as a photosynthetic component. Some of the enzymatic terminology is not universally accepted, e.g., "carboxydismutase" is also called "ribulose phosphate carboxylase" and "xylulose waldenase" is more commonly referred to as "phosphopentose isomerase".

diphosphate [Ru-diP], sedoheptulose phosphate [S-7-P] and glucose-phosphate [G-6-P]) follow closely thereafter. Compounds such as sucrose and glutamic acid are formed relatively slowly. In other plants, e.g., soybean, the pentose phosphates are not discernible until the relatively lengthy time of five minutes, nor is malic acid as prominent in early phases. As a result of these studies, the scheme of Fig. 3 was deduced. The evidence for this scheme is as follows.

Consider the simplified system:



In this cyclic system, if the PGA arises from the Ru-di-P, a diminution of CO_2 should result in an increase of Ru-di-P (in the light). Similarly, a diminution in the reducing power [H], obtained by lowering the light, should result in the accumulation of PGA.

Fig. 4 shows the result of diminution of the CO_2 from a 1% concentration to 0.003%. Prior to the sudden transition, the various compounds were in a steady state (as shown by the horizontal concentration lines of RuDP and PGA). With the sudden change

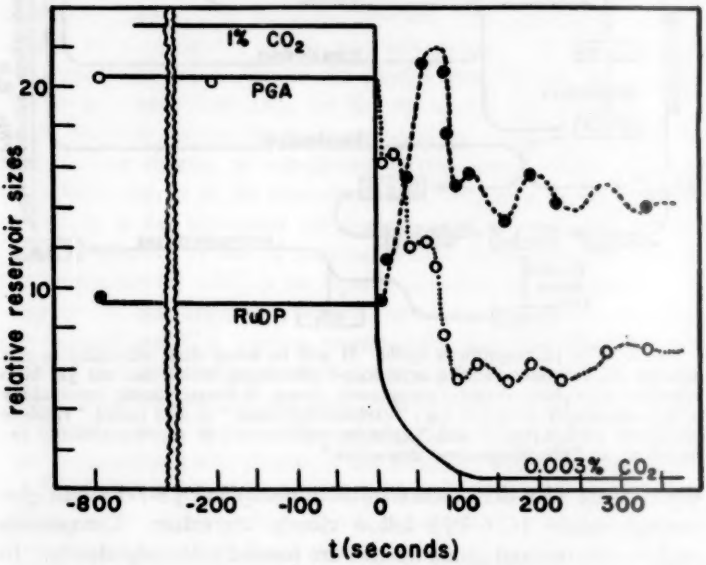


FIG. 4. The change in concentration of phosphoglyceric acid (PGA) and ribulose diphosphate (RuDP) on lowering of the carbon dioxide tension from 1% to 0.003%. The reversal of steady state concentrations of these substances, as well as the lesser changes of other members of the photosynthetic scheme (see original text for details) was used as a basis for elucidation of the carboxylation reaction (see text). Adapted from Wilson and Calvin (84).

in CO_2, only the PGA and RuDP showed major changes. As predicted, the new steady state value of Ru-di-P was increased and that of PGA diminished. [The " overshoot " of RuDP and " false start " of PGA are often associated with transients in steady state systems. The continued oscillations, suggested by the authors as further evidence for cyclic system, may also be associated with linear systems. Their main argument concerning RuDP and PGA appears, however, to be valid.]

The result of sudden diminution in reducing power (that is, cessation of illumination) is shown in Fig. 2. Here the PGA increased (temporarily) in the dark, diminishing with time by conversion to alanine, with a corresponding decrease in the " diphosphates ", presumably primarily RuDP. In more recent studies (12b) the rate of increase of PGA in sudden transition to darkness approximates twice the rate of CO_2 pickup during steady-state photosynthesis. These data (obtained under difficult experimental conditions from only two points) are presented as evidence for the conversion of one mole of RudiP + CO_2 to two moles of PGA. [In Racusen and Aronoff's similar experiments (65), where $C^{14}O_2$ was fed to soybean leaves for 30 seconds directly after the light was turned off, no PGA or GA was found—only alanine. It must be presumed that the reactions in the soybean were proceeding more rapidly than in the algae.]

More recent studies (12b) with *Scenedesmus* by the Berkeley Radiation Laboratory group show marked increases in glutamic, and somewhat less in citric, acid immediately upon termination of a light period.

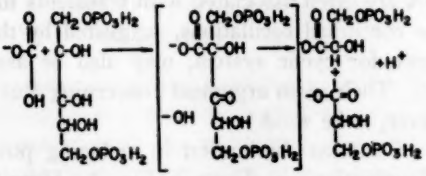
The residual cycle follows from enzymatic carbohydrate reactions, elucidated independently in a number of laboratories. As will be shown, this scheme is essentially in agreement with the distribution of radioactivity within the main classes of compounds.

REACTION 1: The carboxylation reaction

$$(\text{Ru-di-P} + CO_2 + H_2O \rightarrow 2\ \text{PGA})$$

This reaction, which may proceed exergonically (see below), appears to be the sole point at which the carbon dioxide enters the system. (Malic acid, however, may be formed separately by a unique photosynthetic reaction [65]). The enzyme has been iso-

lated (62) and shown to react only with (what is believed to be) Ru-1,5-P (not Ru-5-P, Ri-5-P or F-1,6-P), yielding PGA without discernible intermediates. Calvin suggests the reaction mechanism:



Ru-1,5-P enolate — hypothetical intermediate — 2 PGA⁻ 1

In this reaction, C_3 of the sugar is oxidized, the CO_2 being carboxylated; consequently, Calvin's laboratory calls the enzyme "carboxydismutase". Racker (64) notes that the structure of the substrate is not precisely known, e.g., it may be 3-ketopentose diphosphate instead of 2-keto-. Even so, in this instance identical enolates would be obtained. Pending verification of the mechanism, some groups prefer to call this enzyme "pentose diphosphate carboxylase".

The uniqueness of autotrophic organisms (i.e., photosynthetic and some chemosynthetic) may reside in the presence of this enzyme. It has not been found, for example, in animal tissue.

REACTION 2. $\text{PGA} \xrightarrow{[H]} \text{triose phosphate}$ 2

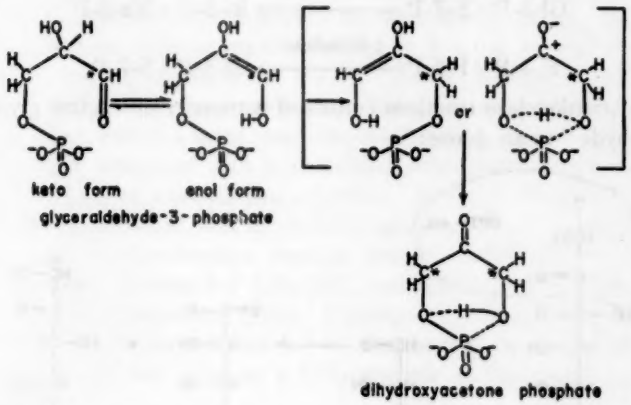
This reaction, being a reduction of the carboxyl to an aldehyde, requires energy. It is one of the two places in the scheme where appreciable energy is required (the other is the phosphorylation which ultimately results in Ri-5-P → pentose-di-P). The enzyme for this reversible reaction is phosphoglyceric acid dehydrogenase which possesses pyridine nucleotide as a prosthetic group. Arnon (4) describes three PGA-dehydrogenases in leaves of higher plants: *a*) a DPN-dependent one requiring P_i, from the 80–90% $(NH_4)_2SO_4$ fraction, Gl-3-P + DPN + $P_i \rightleftarrows$ di-PGA + DPN + H^+, and which is also found in non-green tissues (roots and seeds); *b*) a TPN-dependent one, requiring P_i and obtained from the filtrate of the heated leaf (4.5′, 58°) in the 50–70% $(NH_4)_2SO_4$ and reacting according to the equation: Gl-3-P + TPN^+ + $P_i \rightleftarrows$ diPGA + TPNH + H^+; *c*) another TPN-dependent one, found in the

50–60% $(NH_4)_2SO_4$ dialyzed fraction which is inhibited by P_i: Gl-3-P + TPN$^+$ + HOH ⇄ PGA + TPNH + H$^+$. Only the last is unique to chloroplasts, and its inhibition by P_i explains the inability of earlier workers, using phosphate buffers, to obtain CO_2 fixation with isolated chloroplasts (19).

REACTIONS 3. The isomerase reactions:

Gl-3-P ⇄ DHAP 3a
Ri-5-P ⇄ Ru-5-P 3b

The isomerization of glyceraldehyde to dihydroxyacetone is well known, but the precise mechanism is not yet understood. In particular, the possibility of partial equilibration of C_1 and C_3 must be considered, since this provides the most reasonable explanation of radioactive distribution in certain transketolase reactions (see below). If, in the conversion of Gl-3-P to DHAP, a fraction of the product proceeded thru a symmetrical intermediate, the initial radioactivity which would reside in C_1 of Gl-3-P would be in both C_1 and C_3 of DHAP:



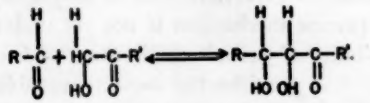
The action of isomerase (as prepared at present) on ribose phosphate is stated to result in a variety of compounds, only a minor fraction of which is ribulose phosphate (64). In principle, if the enzyme involved does not convert ribose to ribulose, then the enzyme in question is not isomerase. However, in practice, whatever the nature of the enzyme, it converts ribose phosphate to a keto-

pentose which, when phosphorylated with ATP, can condense with CO_2 to form 2 PGA.

REACTIONS 4. The aldolase reactions:

$$\text{Gl-3-P} + \text{DHAP} \rightarrow \text{F-1,6-P} \quad \ldots\ldots\ldots\ldots \quad 4a$$
$$\text{E-4-P} + \text{DHAP} \rightarrow \text{S-1,7-P} \quad \ldots\ldots\ldots\ldots \quad 4b$$

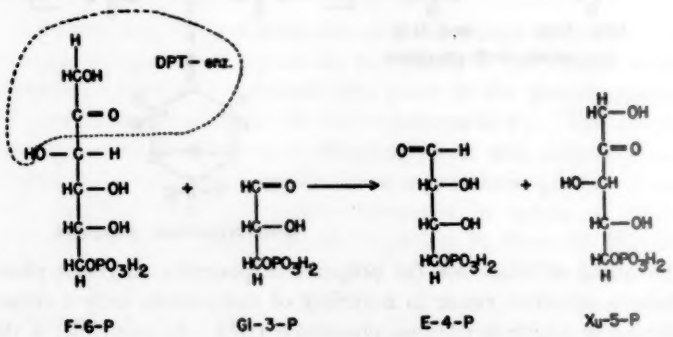
Aldolase is a well known enzyme, having been isolated and crystallized from peas (71). The reaction involves the condensation of a carbonyl and an α-keto carbinol:



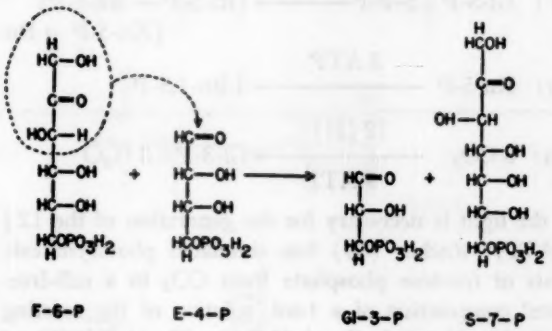
REACTIONS 5. The trans-ketolase and -aldolase condensation reactions:

$$\text{Gl-3-P} + \text{F-6-P} \xrightarrow{\text{t-ketolase}} \text{E-4-P} + \text{Xu-5-P}$$
$$\text{Gl-3-P} + \text{S-7-P} \xrightarrow{\text{t-ketolase}} \text{Ri-5-P} + \text{Xu-5-P}$$
$$\text{E-4-P} + \text{F-6-P} \xrightarrow{\text{t-ketolase}} \text{Gl-3-P} + \text{S-7-P}$$

The transketolase reactions consist of a transfer of "active glycolaldehyde" to an aldose:



The glycoaldehyde is not found free, being transferred as part of the diphosphothiamine (DPT)-enzyme complex. The transaldolase reaction involves transfer of a dihydroxyacetone unit:



No specific prosthetic group is known to be required for the action of the enzyme.

Both types of reactions have been reviewed (64, 41). With specific reference to the photosynthetic scheme depicted, it is apparent that the *minimal* requirements of the photosynthetic cycle would avoid either the aldolase reaction leading to S-1,7-P, or the transaldolase reaction resulting in S-7-P. Either reaction requires E-4-P, *which has not yet been found.* The general explanation for its absence is that of a small pool—the smaller the pool, the less the radioactivity associated with it—resulting either from the rapidity of the aldolase or transaldolase reactions. Secondly, from stereoisomeric consideration, and as verified experimentally (48, 68), the product of the transketolase reaction between S-7-P and Gl-3-P should include xylulose-5-P (Xu-5-P), not ribulose-5-P. This has recently been substantiated (12a). Consequently, a waldenase is required to convert the xylulose-5-P to ribulose-5-P, since Xu-1,5-P apparently will not serve as a CO_2-acceptor in the carboxylase reaction.

A minimal set of reactions involved in photosynthetic CO_2 fixation would then include

a) $3\ CO_2 + 3\ \text{Ru-1,5-P} \longrightarrow 6\ \text{PGA}$

b) $6\ \text{PGA} \longrightarrow 6\ [\text{Gl-3-P} \rightleftarrows \text{DHAP}]$

$$c)\ \text{Gl-3-P} + \text{DHAP} \longrightarrow [\text{F-1,6-P} \rightarrow \text{F-6-P} + P_i]$$

$$d)\ \text{Gl-3-P} + \text{F-6-P} \longrightarrow \text{E-4-P} + \text{Ru-5-P}$$

$$e)\ \text{Gl-3-P} + \text{E-4-P} \longrightarrow [\text{S-1,7-P} \rightarrow \text{S-7-P} + P_i]$$

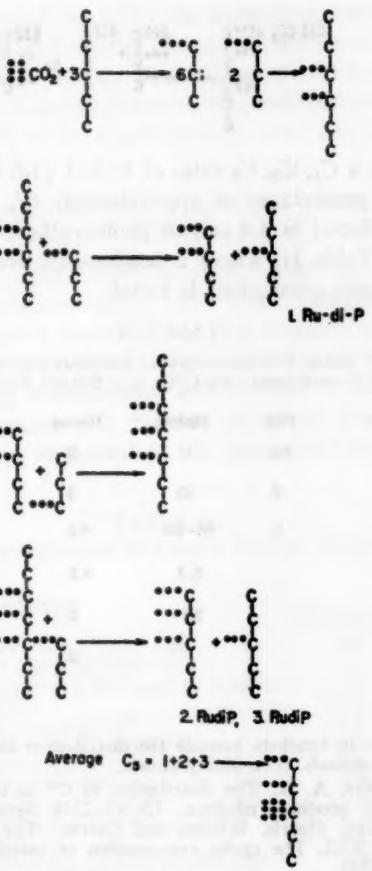
$$f)\ \text{Gl-3-P} + \text{S-7-P} \longrightarrow [\text{Ri-5-P} \rightarrow \text{Ru-5-P}] + [\text{Xu-5-P} \rightarrow \text{Ru-5-P}]$$

$$g)\ \text{Ru-5-P} \xrightarrow{3\ \text{ATP}} 3\ \text{Ru-1,5-P}$$

$$\text{Sum:}\ 3\ CO_2 \xrightarrow[9\ \text{ATP}]{12\ [H]} \text{Gl-3-P} + 3\ H_2O$$

where the light is necessary for the generation of the 12 [H] and the 9 ATP. Racker (64) has simulated photosynthesis by the synthesis of fructose phosphate from CO_2 in a cell-free system. A typical composition of a 1-ml. solution of the reacting system included: 50 μM tris buffer (pH 7.6); 50 μM $KHCO_3$; 0.25 μM Ri-5-P; 2.5 μM $MgCl_2$; 0.5 μM DPN; 1 mg. Gl-3-P dehydrogenase; 65 μg PGA kinase; 280 μg aldolase; 3.5 mg. of a spinach fraction containing: phosphopentose isomerase, kinase, carboxylase, transketolase, transaldolase, and hexose diphosphates; 5 μM glutathione; 40 μM ATP; 1.5 mg. hydrogenase. Gas phase: H_2, 60' 25°. The mixture was assayed for F-6-P spectrenzymically. The hydrogenase in the presence of a hydrogen atmosphere is required to reduce DPN^+. This DPNH then provides the reducing power both for the reduction of PGA and for phosphorylation.

The distribution of initial radioactivity within the components of the cycle is in accord with that postulated by the scheme depicted in Fig. 3. Thus, if we denote the relative amount of activity by the number of dots opposite particular carbons, the expected distribution at the end of the first cycle within key compounds may be obtained approximately from the skeletal formulae as follows:



Consequently, at the end of the first cycle, the Ru-1,5-P formed should have all the activity in $C_{1\rightarrow 3}$, in a corresponding ratio of approximately 1:1:3 (20:20:60 %). Similarly, hexose should be labeled (equally) only in $C_{3,4}$ and sedoheptulose (equally) only in $C_{4,5,6}$, while the PGA or triose should be only carboxyl- and C_1-labeled, respectively. The PGA formed (2nd cycle) by reaction of CO_2 with Ru-1,5-P of the distribution attained in the first cycle:

will then have a C_1, C_2, C_3 ratio of 4:1:1 [12:3:3 = 6:1.5:1.5] or respective percentages of approximately 67, 16.5, 16.5. The actual values found in 5.4 sec. of photosynthesis by *Scenedesmus* are given in Table II, where a semiquantitative agreement with the approximated calculations is found.

TABLE II

DISTRIBUTION IN SOME PHOTOSYNTHETIC INTERMEDIATES OF PHOTOSYNTHESIZING ALGAE (*Scenedesmus*) FED $C^{14}O_2$ IN A STEADY STATE FOR 5.4 SECONDS

C number	PGA	Pentose	Hexose	Sedoheptulose
C_1	82	11	3	2 [33]
C_2	6	10	3	2
C_3	6	64–69	43	28
C_4		5.3	42	25 [8]
C_5		3.1	3	26 [49]
C_6			3	2
C_7				2 [0]

* The numbers in brackets provide the distribution in soybean leaf sedoheptulose in 0.4 seconds (non-steady state).

Source: Zweifler, A. G., The distribution of C^{14} in the carbon atoms of photosynthetically produced ribulose. UCRL-2334, September 1953. Bassham, Benson, Kay, Harris, Wilson, and Calvin. The path of carbon in photosynthesis. XXI. The cyclic regeneration of carbon dioxide acceptor. UCRL-2369 (1953).

An objection to the general scheme suggested above is given by Boichenko and Zakharova (15) whose arguments are based upon an observed variation of product with increasing concentration of CO_2. In their method of analysis, products of short-time photosynthesis may be divided into three fractions: *a*) that in intact chloroplasts, *b*) that in substances precipitable from the residual solution with 60% acetone, and *c*) residual. PGA belongs in the last category. The initial product of fixation is found first in fraction *b*, especially if the leaves are poisoned with hydroxylamine to slow the rate of photosynthesis. With CO_2 concentrations greater

than 0.08%, the initial reactions are accelerated (and subsequent reactions inhibited) to the point where rate studies are believed to be meaningless. The primary product, isolated by boiling fraction *b* in water and precipitating the substance with barium ion in 80% ethanol, is believed to be a polyhydroxy acid not identical with PGA.

THERMODYNAMICS OF THE PHOTOSYNTHETIC CARBON CYCLE

The values of reaction energies of glycolysis and free energies of specific compounds involved have been thoroughly estimated. Calculating analogous reactions and free energies of homologous compounds in photosynthesis (making reasonable assumptions with regard to ring stabilizations, and fractions of the molecules in cyclic form), Bassham (10, 11) has calculated the free energies of the various steps suggested for the photosynthetic carbon cycle (Table III).

TABLE III

ΔF's OF THE REACTIONS OF CARBON DIOXIDE ASSIMILATION

Reaction	ΔF′ (kcal.)	N = times/cycle	NΔF′
1) $HCO_3^- + Ru\text{-}1,5\text{-}P^{-4} \rightarrow 2\ P^{-2}GA^{-1} + H^+$	−9.5	3	−28.5
2) $P^{-2}GA^{-1} + ATP^{-4} + TPNH + H^+ + H_2O \rightarrow Gl\text{-}3\text{-}P^{-2} + TPN^+ + ADP^{-3} + HPO_4^{-2}$	−1.5	6	−9.0
3) $Gl\text{-}3\text{-}P \rightarrow DHAP^{-2}$	−1.3	1	−1.3
4) $Gl\text{-}3\text{-}P^{-2} + DHAP^{-2} \rightarrow F\text{-}1,6\text{-}P^{-4}$	−3.2	1	−3.2
5) $F\text{-}1,6\text{-}P^{-4} + H_2O \rightarrow F\text{-}6\text{-}P^{-2} + HPO_4^{-2}$	−4.8	1	−4.8
6) $Gl\text{-}3\text{-}P^{-2} + F\text{-}6\text{-}P^{-2} \rightarrow E\text{-}4\text{-}P^{-2} + Ru\text{-}5\text{-}P^{-2}$	+3.2	1	+3.2
7) $Gl\text{-}3\text{-}P + E\text{-}4\text{-}P^{-2} \rightarrow S\text{-}1,7\text{-}P^{-4}$	−4.2	1	−4.2
8) $S\text{-}1,7\text{-}P^{-4}\ H_2O \rightarrow S\text{-}7\text{-}P^{-2} + HPO_4^{-2}$	−4.6	1	−4.6
9) $S\text{-}7\text{-}P^{-2} + Gl\text{-}3\text{-}P^{-2} \rightarrow Ri\text{-}5\text{-}P^{-2} + Ru\text{-}5\text{-}P^{-2}$	+1.4	1	+1.4
10) $Ri\text{-}5\text{-}P^{-2} \rightarrow Ru\text{-}5\text{-}P^{-2}$	+0.4	1	+0.4
11) $Ru\text{-}5\text{-}P^{-2} + ATP^{-4} \rightarrow Ru\text{-}1,5\text{-}P^{-4} + ADP^{-3} + H^+$	−3.2	1	−9.6
3 × Sum: $3\ HCO_3^- + 9\ ATP^{-4} + 6\ TPNH + 8\ H_2O \rightarrow Gl\text{-}3\text{-}P + 6\ TPN^+ + 9\ ADP^{-3} + 8\ HPO_4^{-2}$			−60.2

—Adapted from Bassham (10, 11)

To proceed to the free carbohydrate level, e.g., to glucose, two additional steps, occurring one-half time each cycle, are required:

$$F\text{-}6\text{-}P \rightarrow G\text{-}6\text{-}P^{-2}, \quad \Delta F' = +0.1 \text{ kcal.}$$

$$G\text{-}6\text{-}P^{-2} + H_2O \rightarrow G + HPO_4^{-2}, \quad \Delta F' = -4.9 \text{ kcal.}$$

$$\text{Sum: } F\text{-}6\text{-}P^{-2} + H_2O \rightarrow G + HPO_4^{-2}, \quad \Delta F' = -4.8 \text{ kcal.}$$

At one-half unit/cycle, the ΔF′ accumulated = −2.4. To make the

½ F-6-P from the Gl-3-P generated by the cycle, we require one-half the ΔF's of reactions 3), 4), and 5) = ½(− 1.3, − 3.2 and − 4.8) = − 4.6 kcal. Consequently, the total change in free energy involved in the conversion of 6 HCO_3^- to glucose would be 2(− 60.2 + − 2.4 + − 4.6) = − 67.2 kcal. The $\Delta F'$ for an entire molecule would then be − 134.4 kcal.; per molecule of HCO_3^- it would be − 22.4 kcal. This − 22.4 kcal. represents the *excess* of free energy supplied to the cycle by the TPNH–ATP system over that required for its overall operation. The energy required for the reduction of bicarbonate ion to the carbohydrate level is 115.6 kcal., i.e.,

$$HCO_3^- + H^+ \rightarrow [CH_2O] + O_2, \Delta F' = + 115.6 \text{ kcal.}$$

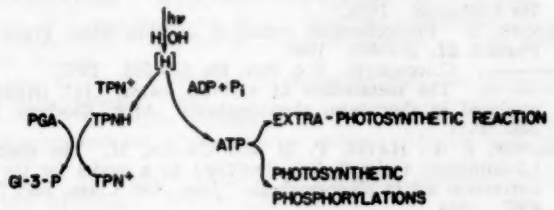
The energy supplied to the cycle, per bicarbonate, is supplied by the hydrolysis of ATP and the oxidation of TPNH. The energy available through the former is $\Delta F = 3(-11.3) = -33.9$. Recent measurements (20, 51) of the $\Delta H°$ of ATP hydrolysis provide values of ~ 4.8 kcal. and estimated $\Delta F°$ of ~ − 6.0 kcal., corresponding to $\Delta F'$ pH = 7 of approximately 7.0 kcal. This will not change the overall qualitative description provided by Bassham's calculations

$$\begin{array}{rll} TPNH \rightarrow TPN^+ + H^+ + 2\,e^- & \Delta F = -14.9 \\ \frac{1}{2}\,O_2 + 2\,e^- + 2\,H^+ \rightarrow H_2O & \Delta F = -36.7 \\ \hline TPNH + \frac{1}{2}\,O_2 + H^+ \rightarrow H_2O + TPN^+ & \Delta F = -51.6 \end{array}$$

For the 2 TPN's involved, $\Delta F = -103.2$, and the combined energy supplied to the system is − 103.2 + − 33.9 = − 137.1 kcal. Consequently the excess of energy supplied over that required is then − 137.1 − + 115.6 = − 21.5 kcal.

The primary evidence for the inclusion of TPN^+ in the photosynthetic scheme is: *a*) the existence of a Gl-3-P-dehydrogenase specifically requiring TPN^+ and not requiring P_i; and *b*) the differential spectral studies of Duysens (1955) where illumination results in the appearance of a band at 350 mμ, corresponding to reduced pyridine nucleotide. However, the latter will also occur merely as a reflection of a general change in the redox potential, or, indeed, even of H^+ concentration alone. The primary evidence for the involvement of ATP, apart from the stoichiometry, results from the studies of Bradley and Calvin (16), Strehler (70) and Arnon (5). The last has divided the major biochemical systems in photo-

synthesis by isolated chloroplasts into a hierarchy of three: *a*) the Hill reaction, which will occur even in those reactions where phosphorylation or carbon assimilation will not; *b*) photophosphorylation, which may occur in those chloroplasts (or grana) where carbon dioxide assimilation will not—but which will certainly permit the Hill reaction; *c*) photochemical carbon dioxide assimilation, which is more sensitive than either the Hill reaction or photophosphorylation. Thus photophosphorylation, which proceeds anaerobically, requires the operation of the Hill reaction, and carbon dioxide assimilation requires both the Hill reaction as well as photophosphorylation. As a consequence, Arnon and his colleagues suggest competition between photophosphorylation and carbon dioxide assimilation for the photochemical energy:



As a corollary, in the presence of excessive P_i, as when phosphate buffer is used for chloroplast isolation, carbon dioxide assimilation is completely suppressed. Thus the inability of earlier investigators to demonstrate the assimilatory capacity of the chloroplasts is in part explained. The residual explanation lies in the ease with which the carboxylase is removed from the chloroplast by washing.

Apart from the individual articles upon which this manuscript is based, the writer is particularly indebted to three recent reviews on photosynthesis: " Photosynthesis " by R. Hill and C. P. Whittingham (Methuen, London, 1955); W. Vishniac's " Biochemical Aspects of Photosynthesis ", Ann. Rev. Plant Physiol. **6**: 115–134 (1955); and R. Lumry's " Photosynthesis ", Ann. Rev. Plant Physiol. **5**: 271–340 (1954). The last contains numerous references, inclusion of which would have exceeded the scope of this article as well as authoritative discussions of the physical chemistry involved. In the middle one is a cogent summary, not only of the

biochemistry of photosynthesis but of its relation to non-photosynthetic systems. The first monograph is characterized by a breadth of view coupled with the brevity which only the most accomplished investigators may hope to attain.

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DETERIORATION OF STORED GRAINS BY FUNGI[1]

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Although deterioration in stored grain typical of that caused by fungi was described more than 40 years ago (17, 45), only recently has the problem become recognized as one of some importance. It is supposed that combine-harvesting of wheat and other small grains has led to increased amounts of seed being stored with a high moisture content. The greater quantities of grain now commonly stored in bulk for two to five years have certainly increased all of the hazards that accompany storage, including those caused by fungi. Realization that fungi are involved in many of the deteriorative processes in bulk stored grains also is relatively new. Often deterioration is subtle in onset and accompanied by no indications of trouble that are apparent to the practical men in the grain trade, and hence losses that we now know to be caused mainly or solely by invasion of the stored seeds by fungi, have been attributed by them to mysterious causes.

NATURE OF LOSSES

Deterioration in stored grains is manifested by: *a*) decrease in germination, important in barley to be used for malting, and in seed to be used for planting; *b*) decrease in processing quality, as in wheat and corn for milling; corn and other seeds used for starch; and flax, soybeans, cottonseed and other seeds used for oils; *c*) sick or germ-damaged grain, a trade and grading term used to designate brown color of the germs, especially in wheat and corn; *d*) heating; and *e*) mustiness. Factors other than fungi may at times be involved in some of these types of deterioration, but fungi can and do cause all of these deleterious changes in stored seeds.

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FUNGI INVOLVED

Somewhat more than 50 species of fungi and a considerable number of species of bacteria have been isolated from agricultural seeds (42). Bacteria do not normally appear to be involved in the deterioration of stored seeds because, so far as known, they require free water to grow, and seeds seldom are stored under conditions where free water is available. There are, of course, some exceptions to this, but to date they appear to be minor in extent and significance.

Fungi in seeds may be rather arbitrarily divided into two groups, designated field fungi and storage fungi. The field fungi are those that invade the developing or mature seed while it is still on the plant. The major genera are *Alternaria, Helminthosporium* and *Fusarium,* in non-weathered seed, plus *Cladosporium, Diplodia, Chaetomium, Rhizopus, Absidia* and a number of others in weathered seed. With the possible exception of *Fusarium,* even heavy invasions of seed by these fungi do not seem to result in decreased storability. We have encountered, however, a few cases in which wheat, with the germs moderately infected by *Fusarium,* developed sick or germ-damaged seeds in storage at moisture contents below those where *Fusarium* originally present could continue to grow. This at least suggests that *Fusarium* spp. should be investigated more thoroughly as possible contributors to one of the common types of storage deterioration in wheat.

The storage fungi are those which develop on and within seeds at moisture contents often encountered in storage, principally *Aspergillus* and *Penicillium.*

Aspergillus glaucus is one of the major fungi that invade stored seeds. This group species is divided into nine " series ", each of which contains a number of subspecies (49). Four of these series —*A. amstelodami, A. ruber, A. repens, A. restrictus*—have been found associated with many cases of deterioration in stored wheat, and appear to constitute the major organisms that invade wheat whose moisture content is 13.2 to 15 per cent, wet weight basis (7, 8, 52). With increasing moisture content above 15 per cent, *A. candidus, A. ochraceus, A. flavus, A. versicolor, A. tamarii* and perhaps a few other species of *Aspergillus,* and *Penicillium* appear. Essentially the same moisture limits prevail for the growth

of these fungi in corn (29) as in wheat. These various species and subspecies of *Aspergillus* differ from one another not only in taxonomic characters but also in such aspects as the moisture content and temperature at which they grow, the speed with which they invade, kill and discolor the germs of the seeds, and the biochemical changes they produce in the seed. Recent unpublished evidence from work in our own laboratory shows that within a given species, strains or varieties differing in these characters may also occur. Thus many of the problems associated with deterioration of stored seeds involve not only a moderate number of species of the ubiquitous *Aspergillus* group but also strains within the species. Some of these species and strains can be isolated from seeds in which they are present only by the use of rather special technics and media.

DETERMINING NUMBER AND KINDS OF STORAGE FUNGI IN SEEDS

It is almost axiomatic in microbiological work that any culture medium used to isolate microorganisms from materials in which they are present is to a certain extent selective, but this very obvious principle has at times been disregarded in work with fungi related to deterioration of stored grains. We have repeatedly found, for example, that acid potato dextrose agar, so widely used in plant pathology laboratories for the isolation of fungi from plant materials, is totally unsuited to detecting the presence of some of the common subspecies of *A. glaucus* that are most prevalent in stored seeds. Also Czapek-Dox agar, a standard medium for some kinds of mycological work and essential for the identification of many species of *Aspergillus*, is of limited value in determining the presence of some of these species of *Aspergillus* in seeds. After tests of 11 different media it was stated (4): "It is obvious —that the standard Czapek-Dox medium—is not suitable for determining the mold count of these samples. It not only gave one of the lowest total mold counts, but—*Aspergillus glaucus* did not appear at all, whereas the other media indicated that this fungus was present in considerable numbers".

No one medium or technic is sufficient to disclose all of the organisms that might be present in a given lot of seeds, and at times it has even been difficult to isolate *Aspergillus restrictus* on agar

media, on which this fungus grows fairly well, and from seeds in which the fungus could be detected by microscopic examination (7). Recently we encountered what appears to be a new species of *Aspergillus* in the germs of nearly 100 per cent of seeds that had been stored for some time at a moisture content of 13.5 per cent; when the seeds were cultured, this fungus grew and sporulated only on the germs, not on the agar; when transferred it grew very slowly on agar media containing 15–20 per cent sodium chloride or 40–80 per cent sucrose, but often did not grow at all on media of lower osmotic pressure. It seems unlikely that even the technics which have been developed to isolate these very xerophytic fungi are adequate to detect them in all cases where they are present in seeds. This is especially true when more rapid growing fungi also are present. In work of this type, as in much other research, the validity of results and of conclusions derived from them depends very greatly upon the technics used and upon the worker's understanding of the limitations and qualifications that attach to the technics. With the use of inadequate technics or of media on which the fungi concerned grow poorly or not at all, it is very easy to conclude that a given sample of seed contains no fungi of significance in storage deterioration, when actually these fungi may be present in large amounts.

While the media and technics of preparation of the seed before culturing are varied considerably in our laboratories, depending on the information desired, several more or less standard media and technics have been developed to determine the number and kinds of storage fungi present on and in seeds. These will be described briefly.

The principal culture medium used is malt-salt agar, consisting of 1–2 per cent malt extract, 2 per cent agar, and 7.5 to 20 per cent sodium chloride. This serves to isolate a larger number of fungi significant in storage deterioration, especially in the *A. glaucus* and *A. candidus* groups, than any other of the media tested (7, 52). For isolation of members of the *A. restrictus* series of the *A. glaucus* group, a medium high in salt or sugar seems to be essential, and even on such media most isolates of most subspecies of the *A. restrictus* series grow very slowly (52). On some media commonly used in microbiological work they do not grow at all. Malt agar, containing 7.5 to 20 per cent sodium

chloride, and Czapek-Dox, containing 40–80 per cent sucrose, have proved fairly satisfactory for isolation of this fungus.

Before culturing, seeds usually are surface disinfected for one half to one minute in one per cent sodium hypochlorite, then rinsed twice in a sterile 7.5 per cent solution of sodium chloride. Implausible as it sounds, even such a light surface disinfection sometimes appears to prevent some of the species of the *A. restrictus* series from growing out of the germs of wheat seed. Sometimes seeds are scattered on the agar without any pre-treatment, or are washed, before culturing, in a jet stream of water, with addition of soap powder or detergent, to remove external dirt and debris. The latter technics have been used especially in determining the fungus flora of supposedly fungus-free seed that was to be used in special tests.

The plates usually are incubated at room temperature, although incubation temperatures of from 10 to 50° C. have been used for special cases. Incubation at 30° C. results in a more rapid appearance of most of the common species of the *A. glaucus, A. candidus* and *A. flavus* groups than incubation at 25° C. It is not unusual to encounter parcels of seeds from commercial storage which contain *A. repens, A. amstelodami, A. chevalieri, A. ruber, A. restrictus* and *A. candidus,* and sometimes most of these may appear from each seed; in those cases the subspecies of *A. glaucus* frequently grow out first, and *A. candidus* appears only after a week or so. If a mixture of *A. restrictus* with other subspecies of the *A. glaucus* group is present, as not infrequently happens, especially in parcels containing some sick seeds, it usually is preferable to culture the seeds on agar media containing 15–20 per cent sodium chloride, as well as on a medium containing 7.5 per cent. In practice we often culture 50 to 100 seeds (25 seeds per plate) on each of several different media; experience has shown that only in this way can we be moderately certain of determining the range of fungi present in the seeds.

To determine the number of viable spores of storage fungi present, five grams of seed are weighed to the nearest seed, on a torsion balance, put in 500 ml. of a sterile solution of 0.2 per cent agar and 7.5 per cent sodium chloride in a Waring blendor, and comminuted for one and one half minutes. Five-ml. amounts are removed from this suspension with a pipette whose end has been

cut and fused to give a larger opening, calibrated to deliver 5 ml. of this rather viscous liquid, and this is added to 45 ml. of the suspension medium of the same composition as that noted above. This is shaken vigorously to distribute the suspended material, then one-ml. aliquots are removed, using either a one-ml. syringe or a one-ml. pipette calibrated to deliver one ml. of this suspension, and placed in each of two or more replicate dishes. Malt-salt agar, melted and cooled to 50° C., is added; the agar is swirled to distribute the suspended material and allowed to harden; the dishes are then incubated.

Agar is added to the suspension medium to give a uniform suspension of spores and seed particles. In extensive tests to determine the sources of variability in numbers of colonies obtained in replicate plates of blendorized seeds, it was found that, when water was used as the suspending medium, the major source of variability was in successive aliquots taken from any given suspension. Adding 0.2 per cent agar to the medium reduced the variability greatly, and with the present technic colony counts on replicate plates commonly vary only plus or minus 5–10 per cent, and frequently less. Salt is added to the suspension medium because in numerous comparative trials it was found that this resulted in higher counts of some of the *A. glaucus* group, particularly *A. restrictus*.

The plates are incubated at room temperature or at 30° C. for two days, then inverted, without removing the cover, on the stage of a stereoscopic microscope, and with the aid of transmitted light the young colonies are counted and their location marked. This is, at times, an unnecessary refinement, but at other times it is desirable because it is not unusual, where even as few as 50 colonies are present per culture plate, for a number of them to arise near each other. Where *A. restrictus* and some of the faster growing subspecies of the *A. glaucus* group, such as *A. repens, A. amstelodami* and *A. ruber,* are all present in considerable numbers, the faster growing colonies may obscure the slower growing colonies of *A. restrictus* if the colonies are not counted in this way. The plates are similarly examined after three days, then left until the colonies can be identified. Frequently it is necessary to transfer colonies to Czapek-Dox agar containing 20 to 40 per cent sucrose to identify them positively.

Essentially the same method is used for determining number and kinds of molds in flour, although with that material no comminution in a blender is required; it has been accepted as standard by the American Association of Cereal Chemists and is included in the latest edition (VI) of Cereal Laboratory Methods (1).

WHERE AND IN WHAT FORM FUNGI OCCUR WITHIN SEEDS

Mycelium is common under the pericarps of wheat seeds (6, 27) and under the hulls and pericarps of barley (53) and rice (16). At the time of harvest, most of this mycelium appears to be that of *Alternaria* or other field fungi and relatively little of storage fungi (6). However, light and superficial infections by storage fungi appear to be so general in cereal seeds that it is difficult to obtain even small amounts of cereal seed that are totally free of storage fungi, or that can be freed of them by surface disinfection or other technics that do not injure the seed, even when the seed is produced in the dryland or irrigated regions of northwestern U. S. One of the problems in separating the effects of storage fungi from the effects of processes inherent in the seeds themselves has been that of obtaining seed that could be stored free of storage fungi for periods of several months to a year. In some cases, to be cited later, we have been able to do this.

WHEN STORAGE FUNGI INVADE SEEDS

Rather extensive tests with wheat and barley over a period of some years indicate that there is no significant invasion of the seeds by storage fungi before harvest (52, 53). This has been so even when the harvest season was moist or when the grain was left in the field, either standing, lodged or windrowed, for as long as two weeks during rainy weather after ripening. Some of these tests have involved wheat grown in various portions of the hard and soft winter wheat regions, from Texas to Oklahoma to New York and Virginia, as well as more extensive tests of grain grown in Minnesota. In no single case have we ever found an appreciable amount of infection of the seed by storage molds prior to harvest.

The reason for this is not certainly known, but there is evidence

that so long as the seeds remain attached to the rachis and surrounded by glumes, they are much less susceptible to invasion by the fungi that cause deterioration in storage than seed that is threshed out (52). As stated above, light and apparently superficial infections by storage fungi are common and in our experience almost universal in wheat and barley; but infections by these fungi heavy enough to account for later deterioration, without more extensive growth by these fungi during the storage period, have not been encountered. Rather extensive invasion may, however, develop within a week or two after harvest if the seed is stored under conditions that permit or promote rapid growth of the fungi concerned.

CONDITIONS THAT INFLUENCE INVASION OF STORED GRAINS BY FUNGI WHICH CAUSE DETERIORATION

A complex of conditions influences the invasion of stored grains by fungi, and in practice these operate together. The major ones are moisture content, temperature, amount of previous infection of the seeds by the fungi concerned, time, and the activities of various grain-inhabiting insects and mites:

MOISTURE CONTENT. Federal regulations (54) specify maximum moisture contents of 14.5 per cent for hard red spring and durum wheats (those with 14.5–16 per cent moisture are graded "tough"); 14 per cent for other classes of wheat (those with 14 to 15.5 per cent are graded "tough"); 14.0 per cent moisture for No. 1 corn, and 15.5 per cent for No. 2 corn. The moisture content limits for wheats presumably were based on studies made in 1918 (2) and in 1925 (11), which indicated that wheat stored at a relative humidity below 75 per cent, or below an equilibrium moisture content of 14.5–15 per cent, did not respire measurably and thus presumably was safe for long time storage. Actually these tests, as well as some later ones, the results of which appeared to support the limits established earlier (33–35), involved storage for only two to three weeks, and the basis for determining whether fungi were developing, or whether deterioration was occurring, was mainly measurement of respiration. Two errors may have been involved: *a*) that tests over a period of three weeks would predict accurately the behavior of grain over a year or more; *b*) that growth of fungi or incipient deterioration could be

detected, at moisture contents of 14–14.5 per cent, by measurement of respiration.

Actually there is no experimental evidence to prove that fungi growing in the germs of wheat whose moisture content is 14 per cent will respire at a rate sufficient to be detected by present technics. During the last five years we have encountered several species of fungi, all of them in commercial lots of seed in which deterioration was progressing, that are able to invade, kill and discolor germs of wheat at moisture contents of 13.5–14.5 per cent. *A. restrictus, A. ruber, A. amstelodami* and the unidentified species of *Aspergillus* recovered from germs of seed mentioned above, all appear to be able to invade and cause deterioration of wheat at moisture contents at or just below the limits now specified for long time storage. The moisture content specified for No. 2 corn (15.5 per cent) is known to permit at least moderately rapid invasion by some species of *Aspergillus* (29), and this is amply supported by unpublished work in our own laboratory. It seems that the present regulations concerning the moisture contents permissible for long time storage of grains are unrealistically high.

Several other complications enter into this. One is the means by which moisture content is determined. In the grain trade, moisture contents usually are determined by means of an electric moisture meter, on a supposedly average sample of the bulk being tested. There is good evidence (40) that moisture meters may at times give a reading as much as one per cent off, even when operated carefully. This appears to be true especially when the moisture contents are in the critical range. If not carefully calibrated and carefully operated, the margin of error may be greater. Recently, for example, the writer obtained a number of samples of soft red winter wheat that had deteriorated in storage during the winter of 1955–56. The moisture content of these samples, as determined by the elevator operator with an electric moisture meter, was 11.9 per cent. As determined in the laboratory, by oven drying according to methods specified by Cereal Laboratory Methods (1), the moisture contents ranged from 13.4 to 13.6 per cent. Apparently the same error crops up in the scientific literature. A 1956 paper (13), dealing with the development of flour beetles in grain, stated that wheat stored in equilibrium with a relative humidity of 75 per cent had a moisture content of 12.2 plus or minus

0.1 per cent. The moisture content in this case was determined by a Steinlite electric moisture meter; there was no mention of its accuracy having been checked against approved oven methods. There is ample evidence (11) that wheat in equilibrium with a relative humidity of 75 per cent has a moisture content of 14.5–15 per cent, or even slightly over 15 per cent, not 12.2 per cent. If an error of that size occurs in laboratory research, one may suppose that errors of equal magnitude sometimes appear in practical grain testing, where many samples are tested hurriedly.

It does not appear to be generally appreciated, especially by those outside the field of cereal chemistry, that moisture content in grains and grain products can be determined accurately only if the methods and equipment specified by those who have established the accepted standards are rigidly adhered to. Moisture content determinations in cereals are not to be taken lightly, and the evidence presented above suggests that data on moisture content determined by any but the approved procedures may often be regarded with suspicion or disbelief.

The use of average samples introduces another fruitful source of error. Granted that grain is bought and sold on a basis of average moisture content; in storing grain, the critical factor, so far as growth of fungi and its attendant deterioration are concerned, is not the average moisture content but the highest moisture content that prevails in any considerable portion of the bulk for any considerable length of time. Evidence from commercial bulks of grain will be instructive here.

In one case, a bin of hard red spring wheat, stored at an average moisture content of 12.8 per cent, began to heat after it had been in storage for three months. As the bin was emptied we obtained a number of samples from the belt near the hopper, put them into moisture-proof cans, and determined their moisture contents by oven drying in the laboratory. Over half the samples so taken and tested had a moisture content of 14 per cent or over, and a few had a moisture content in excess of 16 per cent. In another bin, containing winter wheat with an average moisture content of 13.0 per cent, we obtained samples by probe from depths of five, 15 and 30 feet in each of six different places in the bin, put them immediately into tight containers, and tested their moisture content in the laboratory. Nine of the 18 samples had moisture con-

tents in excess of 13.5 per cent; four had moisture contents just over 14 per cent. The moisture contents of these samples were just above the point where molds might invade the grain, instead of just below, as the elevator operator supposed. In a third case we buried high grade, seed quality wheat whose moisture content was 11 per cent, in small cloth bags at known locations in the bin as the bin was filled. The wheat going into the bin had an average moisture content of 13.2 per cent. Some heating occurred in portions of the bulk after storage for about three months, and the bin was emptied. The bags were recovered on the belt near the hopper, the grain put into moisture-proof tins, and taken to the laboratory for moisture content determinations. About one third of these samples had moisture contents over 14 per cent, several others over 15 per cent. The average moisture content of these samples when the bin was emplied was 13.2 per cent, the same as the average for the entire bulk. Data on mold invasion of these samples indicated that at some time during storage, nearly all of them had for a while been at a moisture content that permitted heavy invasion by *A. glaucus, A. candidus* and *A. flavus*. The samples originally had been almost free of storage molds. The fact that so many of the seeds in so many of the samples had been rather extensively invaded by *A. candidus* and *A. flavus* indicated that these samples had been at moisture contents of 15 to 17 per cent for a long enough time to permit such invasion. The figure on average moisture content was accurate but certainly gave no accurate base for judging risk of deterioration (8).

We now have considerable additional evidence that the practice of testing only average samples for moisture contents gives only a very haphazard picture of what may be happening in some large bulks of stored grain. Closer attention to this and to the accuracy of moisture content determinations undoubtedly would help remove much of the mystery that up to this time has surrounded many cases of deterioration in commercial bins where the grain supposedly was at a moisture content safe for long time storage.

VARIATION IN MOISTURE CONTENT AND TRANSFER OF MOISTURE WITHIN A GIVEN BULK. It is well established that, in any moderately well enclosed bulk of grain in which differences in temperature are maintained for some time, moisture will be transferred from the warmer to the cooler portions of the bulk (37). Thus in

a bulk where the moisture, at time of binning, is uniform throughout the mass of grain, it may become unequally distributed upon storage. There is a greater transfer of moisture with greater differences in temperature than with small differences in temperature between different portions of the bulk. Also, in the cereal grains, if the bulk originally contains a moisture content near the critical limit (i.e., about 13.5 per cent) or above, subsequent moisture transfer from one portion of the bulk to another is likely to be more rapid, and larger in extent, than if the moisture content is lower (around 11–12 per cent). This is explained by the relative humidity–moisture content relationship. As the relative humidity rises from 50 to 65 per cent, the equilibrium moisture content of the grain increases only slightly; as the relative humidity rises from 65 to 80 per cent, the moisture content of the grain increases rapidly (11, 46). In practice this means that if grain is stored at a relatively high temperature and a moisture content near the critical limit, in the fall when there are sharp changes in the daily temperature, temperature differentials are likely to be set up that lead to moisture transfer, mold growth, and to deterioration. If the moist grain is cool when stored in autumn, as often is the case with corn in the northern regions of the United States, no deterioration is likely through the winter. However, with the advent of spring weather and high outside temperatures, especially at the top and on the sunny side of the bins, the grain may be warmed up sufficiently to give rise to convection currents that allow rapid transfer of moisture, and, when the temperature of the moist grain becomes high enough for molds to grow rapidly, sudden spoilage ensues. In many bins, condensation occurs on the inner side of the top during early spring, and the water drips down on the grain in sufficient quantity to wet thoroughly the top layers (44). Often bins are so constructed that leakage may occur from rain, which of course leads to local moist regions (41). Condensation and leakage normally account for only minor wet regions within a large bulk of grain—the greater part of high moisture areas, at least in terminal bins, must be attributed to transfer of water vapor from one portion of the grain to another, with no condensation or leakage being involved.

Some studies now are available to show the pattern of moisture transfer within supposedly typical grain bulks (42); more such

studies are in progress by commercial grain storage firms. Eventually enough data should be available to enable us to know much more exactly than we now do, the circumstances under which such transfer will be great enough to lead to serious spoilage, and to know more accurately its pattern within bulks of different size and shape. Such information would enable us to know the major danger areas within a given bulk under a given set of conditions; assuming that the location of such large-risk or deterioration-prone areas could be known beforehand within a given bulk, it would be a simple matter to remove samples from those areas periodically for testing, and thus anticipate and forestall deterioration without laborious sampling of the entire bulk.

VARIATION IN MOISTURE CONTENT BETWEEN SEEDS. It is common practice, from the farm to the final processor of grains, to blend different lots of grain of different moisture content, to achieve an average moisture content that is within a given grade limit. It is assumed that such mixing leads to uniform distribution of moisture among the seeds so mixed. However, more than 20 years ago (18) it was found that when wheats of different moisture contents were mixed together thoroughly and allowed to come to equilibrium, the seeds originally high in moisture content retained a higher moisture content than the theoretical average. It was stated: "From these five sets of tests the general conclusion is that when wet and dry wheats are mixed in equal proportions and allowed to lie together, moisture is transferred from the wet to the dry. The transference is complete in three days. Equality of moisture is not attained, there being a final difference of about 2% between the wheats".

This problem requires more attention and investigation than has been given to it, if for no other reason than that the practice of such mixing is so firmly established and widespread. Unless the theoretical equilibrium aimed at in such mixing is below the supposed safe limits for storage, some of the seeds, even after thorough mixing, may retain a moisture content high enough to permit invasion of the seeds by molds, and consequent spoilage.

MOISTURE ABSORPTION FROM THE AIR. Grain may pick up moisture from the air relatively rapidly. In some of our own unpublished tests, wheats of 12 per cent moisture were stored in bags

exposed to outdoor air, but protected from rain and snow; the moisture content of these increased to 15 per cent within three months during the fall, and remained so during the winter. According to the Monthly Weather Review, a relative humidity of 75 per cent and above is common during the winter months from Minneapolis to Buffalo. Presumably that portion of the grain exposed to air would come to equilibrium with the relative humidity of the air fairly rapidly. If there is much circulation of the air into and through the bulk, as we know there is in some cases, the grain may gradually accumulate moisture in humid weather. This supposedly is one reason why grain, even though originally dry, is difficult to store without deterioration in humid climates. Moisture content may also be increased drastically in the grain from activities of insects, as will be described below.

TEMPERATURE. Most of the fungi that invade stored grains grow best at temperatures of about 30° C. We have some isolates of *A. flavus,* however, that grow well up to 45° C., and some of *A. candidus* that grow at about 55° C. The growth rate of most of them is greatly reduced below 20° C., but some of the subspecies of the *A. glaucus* group will grow slowly down to 5° C., and *A. restrictus* appears to grow at about the same rate at 15 as at 25° C. Tests aimed at determining the temperature ranges at which these fungi will invade grain should be made with the fungi growing on grain whose moisture content equals that met in practice; tests on agar media often give results that can be applied only to growth of the fungi on agar media. Some strains of *A. repens, A. amstelodami* and *A. ruber,* for example, will not grow on agar media at a temperature above 35° C., but will invade wheat seeds at a moisture content of 15–16 per cent kept at 40–45° C. Much more extensive tests are needed to determine more accurately the relationship of temperature, both constant and fluctuating, to the invasion of seeds by these fungi, and the appearance of commercial damage as a result of such invasion.

TIME. Moisture content, temperature and time are all intimately related to the growth of molds in stored grains. Thus the higher the moisture and temperature, within the limits of growth of the fungi involved, the shorter the permissible storage time. Thus wheat at a moisture content of 14.5 to 15.0 per cent can be

stored safely at 20 to 25° C. for a few months, but not for a year; while at the same moisture content and at a temperature of 10–15° C. it presumably could be stored for a year without serious damage from molds. Apparently the absolute lower limit of moisture content that will permit growth of storage molds in seeds over a period of one to two years at a temperature of 20–25° C. is that in equilibrium with a relative humidity of 70 per cent in the interseed air.

INSECT INFESTATION. There is considerable evidence in the literature to indicate that some of the common grain-infesting insects are able to establish themselves and develop through a normal life cycle in grain whose moisture content is in the range of 10 to 12 per cent (12). However, so far as can be determined by the writer, moisture contents of grain have not been determined after the insects have become established. In some of the tests, moisture content has been determined by the use of electric moisture meters that apparently were not accurately calibrated; a paper already cited, for example (13), dealing with moisture content in relation to growth of the confused flour beetle (*Tribolium confusum*), states that wheat in equilibrium with a relative humidity of 75 per cent had a moisture content of 12.2 plus or minus 0.1 per cent. Wheat in equilibrium with a relative humidity of 75 per cent should have a moisture content of 14.8 to 15.0 per cent, as was established long ago (11) and amply supported by many recent tests. Thus even at the beginning of the tests reported with this beetle, the moisture contents given presumably are in error; either the relative humidity was lower than stated or the moisture content was several per cent higher than stated. The moisture content at the end of the tests was not determined.

In recent cooperative work between the Departments of Entomology and Plant Pathology and Botany at the University of Minnesota with grain insects, such as the granary weevil (*Calandria granaria*), and the lesser grain beetle (*Rhizopertha dominica*), establishment of the insects in grain having an original moisture content of 12 to 15 per cent resulted in rather rapid increases in moisture content to an amount of several per cent. It also resulted in very rapid increase in some of the common storage molds

associated with the development of sick wheat, especially *A. restrictus*. The relationship between some of the insects and mites known to infest grain and grain products, and the fungi known to cause damage of one kind or another to these products, apparently may offer a rather fruitful and hitherto unexplored field of research. It is anticipated that work now under way at the University of Minnesota, dealing with these relationships, will be continued. At present it seems very probable that even moderate infestations by insects in commercial bulks of grain may very greatly increase the hazard of deterioration from fungi, within and probably even at some distance from that portion of the bulk where the insects are established.

OXYGEN–CARBON DIOXIDE RATIO. In agar cultures a 30 per cent concentration of carbon dioxide stimulated *Penicillium*, and a concentration of 40 per cent was required to inhibit the germination of spores of *A. repens* (20). From results of tests with wheat stored at 18 per cent moisture and under different concentrations of oxygen and carbon dioxide, it was concluded that: ". . . mold growth, germ damage, fat acidity, and respiration rate all gradually decreased as the oxygen concentration was lowered. Some mold growth occurred at 0.2 per cent oxygen but the wheat maintained its viability; . . ." and " In the presence of 21 per cent oxygen, increasing levels of carbon dioxide had little effect on respiration rate until the concentration exceeded 13.8 to 18.6 per cent, when a very sharp and marked inhibition of respiration, mold growth, and development of fat acidity occurred. At large carbon dioxide concentrations (50 and 79 per cent), the viability of the wheat remained high and there was little or no germ damage" (39). The little evidence available indicates that even when molds are actively growing on and in seed in commercial bins, the composition of the interseed air remains about the same as that of outside air (41). So far as is known, no tests have been made to determine the effect of carbon dioxide concentrations on the fungi that cause deterioration of stored seeds, at moisture contents of 13.5–15 per cent, and for a period of several months to a year, which are the conditions under which deterioration often occurs in practice. This is rather important, especially since attempts now are being made to control or reduce deterioration in stored grain

by keeping the grain in an atmosphere high in carbon dioxide (26, 49).

PREVIOUS INVASION BY STORAGE MOLDS. If seeds are moderately to heavily invaded by the fungi known to cause deterioration, then dried to a moisture content of 12–14 per cent, deterioration, as indicated by increase in fatty acids, decrease in non-reducing sugars and increase in amounts of "sick" wheat, will continue to develop (21, 47). This "carryover" effect has been observed only in laboratory tests, but it may help to explain some cases of deterioration of grain stored at supposedly safe moisture contents, since it is not unusual to find parcels of grain going into terminal storage with invasions by storage molds as heavy as those in some of the tests cited. Information concerning degree to which given parcels of grain have been invaded by the fungi known to cause deterioration, if obtained at intervals from harvest on, might well give more information on storability and deterioration hazard than any other tests now available.

EFFECTS OF STORAGE MOLDS ON SEEDS

Fungi growing in stored seeds can reduce germination of the seed, cause darkening of the germ or of the entire seed, increase the fatty acid content, cause or contribute to other biochemical changes, increase the moisture content of the seed, and, in extreme cases, cause heating. Each of these will now be discussed.

REDUCTION IN GERMINATION. Seeds of wheat and barley free of fungi that cause storage deterioration can be stored at moisture contents of 15 to 18 per cent and a temperature of 20–25° C. for weeks or months with little reduction in germination (21, 34, 52). This is fairly conclusive evidence that processes inherent in the seeds themselves are of very minor significance in death of seeds under those conditions; and it is good presumptive evidence that processes inherent in the seeds themselves are of minor significance in such deteriorations as are encountered in practice. There is an abundance of evidence that invasion of the germs of the seeds by those species of *Aspergillus* encountered in seed that has undergone deterioration, will result in rapid death of the germs (21, 52). These fungi, therefore, are at least facultative parasites. They differ from one another in their degree of pathogenicity, *A.*

restrictus, A. candidus and *A. ochraceus* apparently being more pathogenic than *A. repens.*[3]

Different isolates of *A. candidus* differ greatly from one another in the rate at which they kill wheat seeds at moisture contents of 16 to 17 per cent, and it seems likely that such pathogenic races occur within all major species of seed-invading fungi. The nature of this pathogenicity is mostly not understood; however, *A. flavus* is known to produce toxins that are lethal to wheat seeds (51).

DISCOLORATION OF THE GERM. Darkening of the pericarps toward the point of attachment of wheat seeds may be caused in the field, before or during ripening, by bacteria or by various fungi, for example, *Alternaria, Helminthosporium* and *Cladosporium,* which have dark mycelia and which sometimes invade the pericarps of the seed heavily during humid weather (23, 24, 31). This is quite distinct from the blackening of the germ itself, which is designated in the trade as "sick" or "germ damaged" wheat. Federal regulations specify the maximum percentage of damaged kernels permissible in each grade, and thus sick or germ-damaged seeds constitute a grading factor and a price factor in wheat, indeed in all cereals, including corn.

Development of dark germs in stored seeds was long considered by those in the trade to be caused by mysterious factors. Unfortunately, perhaps, fungi had been more or less excluded as a possible cause; first, on a basis of logic (whose premises were faulty), and second, on a basis of examination of sick seeds with the unaided eye by those entirely unfamiliar with fungi (48). Sick or germ-damaged seeds are detected only by removing the pericarps that cover the germ, to expose the germ surface. Examination of such seeds with the unaided eye is scarcely a method to which a mycologist would give any credence in determining whether fungi are or are not involved.

In recent years it has been found that the germs of seeds that have become sick in commercial storage are almost invariably heavily invaded by storage molds. In many cases the fungi cover the germ surface with masses of mycelium and spores. Inoculated into seeds which then are stored at moisture contents that permit

[3] G. C. Papavizas. Unpublished evidence, Dept. of Plant Pathology, University of Minnesota.

the molds to develop, these fungi cause typical sick seed (52). There now seems no doubt that, in practice, sick wheat commonly is produced by invasion of the germs of the seeds by storage molds. In the laboratory, what appears to be typical sick wheat (judged by brown or black color of the germs) has been produced by storing seed under carbon dioxide, under nitrogen, at temperatures of 35° C. or above and by overexposure to fumigants. Sick seed so produced is not likely to have the biochemical properties—high fatty acid content especially—of the sick seed encountered in commercial storage, and of the sick seed produced as a result of invasion by fungi. It has been supposed that there may be a number of types of sick wheat, one caused by invasion by fungi, others by other factors. All of the evidence from hundreds of samples obtained from commercial bins over the past few years, however, suggests that sick wheat normally is a product of invasion of the germs by species of *Aspergillus;* if other factors ever are involved, they have not yet been detected, and they presumably are of minor significance in producing sick wheat in commercial bulks of wheat. Recent evidence with corn indicates that there, also, invasion of the germ by fungi is the principal cause of development of dark germs. Had the germs of sick seed been inspected with the aid of a stereoscopic microscope and by men acquainted with fungi, when this sort of damage first became an important factor in grading, some 20 years or more ago, it is very likely that molds would at that time have been at least suspected to be a cause of the trouble.

BIOCHEMICAL CHANGES. A variety of biochemical changes accompany or follow invasion of stored seeds by fungi (3, 30, 32–34, 41). The more obvious are increase in fatty acids, increase in reducing sugars, decrease in non-reducing sugars, and increased respiration. At one time these changes were assumed to be produced mainly or solely by the activity of seed enzymes, but as microbiological and biochemical studies were undertaken together, the importance of fungi was gradually recognized, and now these changes are attributed mainly or solely to fungi. As stated recently (5), "The fat acidity and sugar values . . . show that the changes in these properties upon storage are associated with the growth of microorganisms rather than with the metabolic activity of the wheat itself".

Attempts have been made to use the quantity of fatty acids pres-

ent as a measure of deterioration and of grain quality. In rather extensive tests with stored corn it was found that if moist stored corn was aerated there was a fairly good correlation between increase in fatty acids and increase in storage molds (22), at least for so short a time as three weeks. However, if the corn was stored under essentially anaerobic conditions, certain molds increased without any appreciable increase in fatty acids. Different storage molds convert fats into fatty acids at different rates, and some consume fatty acids, being able to use them as their sole source of carbon (22). For these reasons it seems doubtful that the amount of fatty acids in a given sample would furnish any accurate evidence as to its microbiological history.

Almost no studies have yet been made on biochemical changes induced by some of the most common and prevalent storage molds in the *A. glaucus* group which grow in grain at moisture contents of 13.5 to 15 per cent. It is hoped that work eventually will be undertaken to determine some of the outstanding physiological characteristics of *A. restrictus, A. repens, A. amstelodami* and *A. ruber* under conditions that approach those at which these fungi grow in bins.

INCREASE IN MOISTURE CONTENT. As molds grow in stored grain they convert into water a part of the material they consume. In large bulks of grain this means that if molds are growing even slowly in certain portions of the bulk, they will contribute some moisture to the portion of the bulk where they are growing or, if the moisture is transferred by convection or diffusion, to other portions of the bulk. Even in laboratory tests in which moist grain in small lots is continually aerated with air of a given relative humidity, that is, in equilibrium with the original moisture content of the stored grain, the moisture content of the seed is likely to increase as the seed is invaded by molds (34). We do not know whether this is a factor of importance in commercial bulks, but it would be desirable to find out.

HEATING. The fact that microorganisms growing in organic materials can cause an increase in temperature was known before 1900 (10). Rather extensive tests of the growth of fungi, yeasts and bacteria in relation to the heating of materials, such as corn meal and straw, were made in the 1920's (28) and showed that

rapidly growing microorganisms are capable of producing large amounts of heat. As long ago as 1912 there was fairly conclusive evidence (14) that moldy seeds produce more heat than non-moldy seeds when moistened sufficiently to germinate. In 1930 (19) molds were shown to cause heating of stored corn; this is amply substantiated by later work (9). Studies such as these apparently were unknown to, or disregarded by, practical men interested in grain storage, and it was widely believed that heating which occurred in the absence of insects was caused by the grain itself. Later studies with adiabatic devices (34, 41) proved that the respiration of the seeds themselves, at moisture contents below those necessary for germination, and at moisture contents ordinarily encountered in storage, does not contribute significantly to heating. To quote a modern view (5): "The data indicate that the heating of moist wheat in storage can be entirely accounted for by the energy released in the respiration of fungi present on and in the wheat kernels".

Heating of bulk stored seeds is caused by rapid increase in the quantity of certain insects among the seeds up to a temperature of 105 to 110° F. (about 40 to 43° C.), above which the insects cannot survive (12). Where insects have been eliminated, as is true of much grain stored in terminals, the sole cause of heating is the growth of storage molds in the grain.

Detectable heating often is considered by those who store bulk grain as the first evidence of deterioration, and commercial operators often assume that if a given bulk of grain is not heating (according to their methods of detecting heating) it is not deteriorating. This is not necessarily true.

Relatively dry grain is a good insulator, about one third as good as cork (37). Thus any heating that may occur is detectable only if it occurs in the immediate vicinity of the temperature-sensing element. In stored hay (38) the temperature in one portion of a bulk only one meter from the thermocouple was 90° F. higher than that at the thermocouple. Temperature detection cables often are at or near the center of bins; in a bin 15 feet in diameter, 25 per cent of the grain is in the outer foot, and 45 per cent of the grain is in the outer two feet. In a bin 21 feet in diameter, 18 per cent of the grain is in the outer foot, and 34 per cent in the outer two feet; and in a bin 30 feet in diameter, 13 per cent of the grain

is in the outer foot, and 25 per cent in the outer two feet. In the outer portions of many bins rather drastic changes may occur, and considerable deterioration develop, before there are temperature changes at cables near the center.

Also, heating is not always, nor perhaps even usually, a good criterion of deterioration of stored grain by molds. Most of the deterioration caused by molds in commercial storage probably occurs without any detectable heating. For example, we stored wheat at moisture contents of 14 to 16 per cent in vacuum bottles wrapped in a layer of insulation about a foot thick, and there was no detectable rise in temperature within the grain, although it was slowly invaded, killed and discolored by *A. restrictus, A. repens, A. ruber* and *A. amstelodami*. At these moisture contents the fungi grew too slowly to produce any detectable rise in temperature. The actual heat and moisture produced by these fungi growing in wheat at a moisture content of 14 to 15 per cent never have been measured. We do know, however, from actual tests in bins, in which samples have been taped onto temperature cables when the bins were filled and recovered when the bins were emptied, that severe deterioration of the grain may occur without any detectable rise in temperature. Heating is likely to be the final and violent effect of mold invasion of the seed, not an indication of beginning deterioration. There are also other complications. It recently was stated (5): "*A. glaucus* sometimes produced different rates of respiration and heating on this moist dead wheat. These differences were associated with the type of spores produced. Production of ascospores was associated with moderate oxygen uptake and very little heat production. The appearance of conidia, on the other hand, coincided with higher respiratory measurements and more heating". As ordinarily measured in stored grain, heating is, in many cases, less a warning of impending trouble than proof that trouble already has developed.

CONTROL OF MOLDS

The three general methods that have been suggested or tested, either in the laboratory or in practice, to control the growth of storage molds in moist grain have been the use of fungicides, storage under toxic or inert gases, and drying the grain to reduce the moisture content to a safe level.

FUNGICIDES. There is some evidence that certain fungicides (chlorinated phenols), when added to hay in concentrations not obviously toxic to domestic animals, will control, or at least reduce, the molding of the hay (15). Even if this were permissible, adding chlorinated phenols to grain probably would not add to the sales appeal of the products. More than 100 fungicides were tested on wheat stored in the laboratory at moisture contents of 16 to 25 per cent (35); most of those that controlled the molds killed the seed; some of them reduced external growth and sporulation of the fungi and thus kept the seed outwardly clean, but allowed molds to grow internally; some had a differential effect on some of the molds, inhibiting certain species but not others. It has been claimed (40) that microbiological activity in moist stored seed was completely eliminated by treating the seeds with Ceresan-M (ethyl mercury phosphate, 3 per cent; inert materials, 97 per cent). No details were given as to how the seeds were tested for molds after storage. We have encountered cases in which a fungicide applied to seeds did not suppress molds on and in the seed during storage, but, when the seeds were cultured on agar afterwards, the fungicide prevented the fungi from growing out onto the agar. That is, the fungicide was not toxic to fungi on the stored seeds but was toxic to them on agar. Fungicides that depend for their effectiveness upon being dissolved in water are not likely to be toxic at seed moisture contents of 15 to 20 per cent because no free water is available. In tests of the effect of fungicides on molds it is essential to inactivate the fungicide, at the end of the storage tests, before the seeds are cultured. Evidence is now available (36) that Ceresan-M applied, in the concentrations reported above, to seeds that are stored for some time, has no fungicidal effect whatever at moisture contents below those where free water is available. This is supported by unpublished work in our own laboratory.

STORAGE UNDER TOXIC OR INERT GASES. Storage under carbon dioxide has been proposed as a relatively easy means of controlling the growth of molds in stored grain; it has been used in England (26) and France, and to some extent for rice in this country (49). A considerable body of data will be required before we are in a position to determine under what circumstances such storage will be effective and practicable.

At present the only effective method for control of molds in long-time storage is to keep the grain at a moisture content so low that no portion of the bulk is moist enough to permit damaging invasion by storage molds. As indicated above, some hazards may be involved in mixing lots of different moisture content to achieve a theoretical average. Also moisture may, for one reason or another, become unevenly distributed in the bulk of grain with time, or the grain, once dry, may pick up water. Thus control of moisture content is not simply a matter of drying the grain and leaving it in dead storage. Data should be obtained on such grain at intervals and in various portions of the bulk, to determine whether changes in moisture content, mold development, and biochemical and processing characters are taking place, which are of sufficient magnitude to endanger the quality. Under present practices much grain is needlessly shifted from one bin to another, while some which should be shifted is not. Tests that would furnish data on some of the essential characteristics within the bulk would permit more rational and economical handling of grain.

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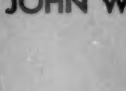

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